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MAX RUBNER

(1854 - 1932)



MAX RUBNER

MAX RUBNER

(JUNE 2, 1854 — APRIL 27, 1932)

"Great men are very rare. They are worth knowing. They give impulse and stimulus to lesser men. They make the world more worth while for others to live in because of their presence in it. Max Rubner was the greatest man I ever knew." These are the words of Graham Lusk in a tribute to Rubner before the American Association for the Advancement of Science on June 23, 1932. One of the elements of Rubner's greatness was his ability to derive broad fundamental concepts from relatively simple experiments.

A native of Munich, Rubner started his scientific career as a pupil of Carl Voit in his early twenties. These were stirring times in the Munich laboratory. The new apparatus of Pettenkofer and Voit for accurately measuring the expired CO_2 of human subjects or experimental animals was rapidly changing the older theories of food metabolism. The major interest in Voit's laboratory at this time was centered around the study of energy transformations in the living body. From the determination of expired CO_2 and the carbon and nitrogen excreted in the urine, the energy metabolism of the fasting man was calculated in terms of grams of fat and protein oxidized in the body. Rubner's early contribution to the problem was the demonstration from bomb calorimeter determinations that about 25% of the total heat value of protein is lost to the body by the excretion of incompletely oxidized nitrogenous material in the urine and feces. His early data, published in 1879, are the basis for the present methods of calculating respiratory metabolism.

Voit had suggested a study of the interchangeability of fat and carbohydrate as sources of body energy. From experiments on fasting rabbits, Rubner in 1878 noted that protein

could replace fat as a source of energy when the fat stores in the body were exhausted. Extending these investigations he conceived the isodynamic law, "that the food-stuffs may under given conditions replace each other in accordance with their heat-producing value." His standard values of 4.1 cal. per gram of protein, 9.3 cal. per gram of fat, and 4.1 cal. per gram of carbohydrate have had world-wide use in the calculation of the food and nutritional requirements of large populations.

With these new caloric values at hand, he began a recalculation of the existing data from metabolism determinations on man and on animals. Meeh had just published (1879) a simple formula for calculating surface area ($S.A. = KW^{2/3}$) substituting weight for volume, and introducing a constant (K) to correct for the varying body shapes from one species to another. The calculations showed that the 24-hour metabolism of Pettenkofer and Voit's fasting man per square meter of body surface was approximately equivalent to that of a man on a medium diet or of a fasting dog or a breast-fed infant.

This was the discovery of Rubner's "law of surface area," that the heat value of the metabolism of the resting individual is proportional to the area of the surface of his body. In my opinion it is his outstanding scientific achievement, both for its breadth of concept and its stimulating influence on research in metabolism, calorimetry, and nutrition. The stature of the concept may be measured by the fact that after more than half a century the subject is still a controversial issue.

During the period of these early investigations, a conflict of ideas developed between the great master and his youthful pupil. In 1881 Voit published his belief that "The unknown causes of metabolism are found in the cells of the organism. The mass of these cells and their power to decompose materials determine metabolism." Voit concluded from his studies of respiratory metabolism that there is an inherent rate of metabolic activity in the body cells which is augmented by the quantity and type of food material (protein, fat or carbo-

hydrate) brought to the cells in the blood stream. Environmental conditions or the need for energy are controlling factors but "cannot possibly be the cause of metabolism."

Influenced by his earlier work on the isodynamic law, Rubner was convinced that the study of energy changes in the body would produce the most rapid advances in basic knowledge of nutrition and metabolic processes. Despite Voit's dissension and the delay in publication until 1883, Rubner held firmly to his theory that the fundamental metabolism of a warm-blooded animal is always constant, and that the increased heat production after the ingestion of food is due to intermediary reactions superimposed on the fundamental level. Their opposing views were never reconciled.

The opportunity to produce experimental evidence for this theory came after he moved to Marburg in 1885 to become the first Professor of Hygiene, joining the notable company of Albrecht Kossel, Hans Horst Meyer, and Friedrich von Müller as a full-fledged independent investigator. Here in his own laboratory in 1889 he constructed, mainly by his own efforts, the first accurate respiration calorimeter. Among his initial experiments was the demonstration that the law of the conservation of energy applied to the living animal body, in that the heat loss from the body agreed with that calculated from the food materials oxidized. In a dog living in the calorimeter for 45 days the total calorimetric measurement of heat production was 17,349 cal. and that calculated from the respiratory metabolism and nitrogen excretion was 17,406 cal.

In 1891 Rubner was invited to Berlin to take Robert Koch's place as Professor of Hygiene. Then in 1909 he succeeded Engelmann as Professor of Physiology, a position he occupied with distinction until he became emeritus in 1924. In the decade following the move to Berlin, the field of energy metabolism was vigorously explored; for example, 50 papers appeared in the *Archives für Hygiene* during this period. Studies of the agreement between the direct measurement of heat production and the respiratory metabolism were extended to

cover a variety of nutritive conditions and diets. A large amount of evidence was accumulated in support of the surface area law in mammals, ranging from the horse to the mouse. The effect of changes in environmental temperature on the metabolic rate was widely investigated, resulting in Rubner's well-known chart showing the areas of physical and chemical regulation of body temperature. Much effort was given to the search for an explanation of the extra heat production caused by the ingestion of food, particularly protein, a reaction which he called "specific dynamic action." The extensive experimental evidence related to these basic concepts which Rubner had obtained during this period was collected and published in 1902 in his comprehensive book, "Die Gesetze des Energieverbrauchs bei der Ernährung."

New interests in the following years continued to produce new concepts. He demonstrated that the energy from mechanical work during exposure to cold would replace the rise in metabolism (chemical regulation) found in the quiescent individual. Investigation of the energy requirements in growing animals led to the conclusion that "The amount of energy (calories) which is necessary to double the weight of the newborn of all species (except man) is the same per kilogram no matter whether the animals grows quickly or slowly." Attention was turned again to the sparing action of carbohydrate on protein metabolism. His eminent pupil, Karl Thomas, demonstrated in man with a starch-cream diet a minimum urinary nitrogen excretion of 2.2 gm daily, which Rubner designated as the minimum or "wear and tear" quota of protein metabolism. During World War I Rubner and his assistants were called upon by the German government to test a large variety of bread substitutes and modifications. Determinations of the fecal loss in nitrogen and calories in man and animals showed that none of the proposed changes gave a product nutritionally equal to white or rye bread.

To attempt to trace the impact of Rubner's pioneering work on the various fields of modern research involving energy metabolism would be a monumental task. Only a few out-

standing examples can be mentioned. The credit for the development of calorimetry in relation to respiratory metabolism undoubtedly is due to Rubner's foresight and persistence. Atwater, who worked in Voit's laboratory with Rubner, was the first to bring the technique to this country. He started the first human calorimeter in 1892 at Wesleyan University, in Middletown, Connecticut, in collaboration with Rosa, the physicist. Francis G. Benedict, working with Atwater, added improvements to the calorimeter at Wesleyan, and then in 1908, as director of the Nutrition Laboratory of the Carnegie Institution of Washington and with the assistance of Thorne M. Carpenter, built the well-known calorimeters for human and animal study in Boston.

Graham Lusk, a pupil of Voit and a close friend of Rubner, also brought the Munich influence to this country. When Lusk became Professor of Physiology at Cornell University Medical College, funds were provided for the construction of a respiration calorimeter large enough to study the metabolism of babies and dogs, which was completed in 1912. The next year Lusk was able to extend the research to the clinic through the installation of the Russell Sage Institute of Pathology calorimeter in Bellevue Hospital under the medical direction of Eugene F. DuBois.

Important as the calorimeter was in the advance of the science of energy metabolism, Rubner considered it only as a valuable laboratory tool, useful in expanding the broad problems of energy exchange in living matter. In a paper published at the time of his retirement in 1924, Rubner states that he considered the concept of the law of surface area as his most important contribution. He lists the discoveries resulting therefrom as follows: the isodynamic law and the caloric basis of metabolism; the physical regulation of body temperature; the specific dynamic action of foods; that metabolism in youth is essentially a surface area phenomenon; that in changes in bodily condition, such as starvation, the surface area law does not apply.

General adoption of the surface area principle as the most satisfactory method of comparing metabolism in humans was stimulated by DuBois and co-workers starting about 1914. Carefully controlled experiments had established the agreement between calorimeter and respiratory metabolism determinations for experimental periods shorter than those used by Rubner and Atwater. Benedict had developed accurate respiratory apparatus which was gaining increasing application in the clinic. Rubner's early data on the comparison of 24-hour metabolism among various species gave obviously high values per square meter since the influence of food and activity was not excluded. Thus it became apparent that conditions of minimal stimuli must be fulfilled to obtain reproducible and comparable results with human subjects. These conditions became the criteria for what was designated as "basal," "standard," or "postabsorptive" metabolism.

In the next two decades an extensive accumulation of basal metabolism data provided the figures for the modification of Meeh's surface area formula to include height as well as weight (DuBois). The Harris-Benedict prediction tables for normal basal metabolism also included age and sex. Respiratory metabolism studies spread widely and rapidly both in clinical research on abnormal metabolism, and in the extension of Rubner's discoveries of the influence of environmental temperature, food, growth, age, and nutritional condition of the body on normal metabolism. Calories per square meter per hour became the generally accepted method of expressing human metabolism, although its general application to animal metabolism data encountered many difficulties, particularly in an accurate comparison among different species. Thus a causal relationship between surface area and basal metabolism became a matter of considerable controversy among the authorities in human and animal physiology.

Rubner continued his wide range of interest in the subject, collecting information on the surface area-metabolism relationship in birds, aquatic animals, amphibians, and reptiles and on the chemical analysis and heat of combustion of their

dried tissues. Shortly before his death he vigorously defended the surface area law before the Prussian Academy of Science in which he was honored as co-secretary with Planck. It should be remembered that Rubner was dealing with fundamental metabolism and surface area in broad terms. No one questioned the evidence that in comparing a range of animals from the largest to the smallest the metabolic rate corresponded in general to the anatomical surface of the body rather than to body weight. But to the nutritionist dealing with agricultural animals, it is practical and accurate to measure body weight whereas surface area measurements are difficult and inexact. The available data have been analyzed by both Brody and Kleiber. Respectively, they recommend that body weight to the 0.7 power or to the 0.75 power be used as the reference unit for "metabolic body size" or "physiologically effective body size." The accuracy is sufficient for comparison between species of animals but is questionable for intraspecific use.

Since the passing of Rubner and Lusk the metabolic concept of surface area has progressed away from the anatomical toward the physiological interpretation. Extended research on heat loss and skin temperatures under various conditions has shown that several overlapping body surfaces are concerned in removing heat from the body. For example, the effective radiation (Bohnenkamp) area is smaller by 20 to 35% than the total surface area, depending upon body posture. Likewise the convection and evaporative areas are variable and difficult to define. Recent research on thermal stress has served to indicate the complex integrated reactions of the peripheral and central nervous systems and of the endocrines in maintaining thermal homeostasis with a minimum of deviation from a balance between heat loss and heat production. It seems quite possible that the growing interest in thermoregulation and heat loss may uncover the interplay of fundamental physiological mechanisms which will vindicate Rubner's faith in a broad causal relationship between surface area and basal metabolism.

The other discovery to which Rubner's name is most frequently attached is that of the "specific dynamic action" of foods, the increase in heat production which follows the ingestion of fat, carbohydrate, or protein, and is quantitatively different for each of these foodstuffs. In his early experiments Rubner saw that protein was much more potent than fat or carbohydrate in increasing the heat production of the body. He then tried the effect of meat at different environmental temperatures and found that the extra energy of specific dynamic action was lost from the body as extra heat when the room temperature was 30°C., but was used to keep the body warm when the room temperature was lowered to 4°C. On the contrary the extra heat from ingested meat could not be used for muscular work. The following experiments in 1910 demonstrated this fact. A fasting man performing 100,000 kg-meters of work increased his heat production 45% over the resting level. Without muscular work a protein diet raised the metabolism 27%. The protein diet plus the same work gave a 70% increase in heat production, thus showing the additive effect of specific dynamic action and muscular work. Rubner postulated that two specific forms of energy are released in the metabolism of protein, one which supplies energy directly for the maintenance of cell life and the other which is the free heat of intermediary thermo-chemical reactions. He never changed his belief that specific dynamic action is due to the heat production of intermediary metabolism. During Lusk's last visit with him in 1930 he refused to elaborate the concept but replied, "There are various possibilities."

Through Rubner's influence Lusk initiated his studies of specific dynamic action and published the results in the second paper (1912) of the Animal Calorimetry series which he and his colleagues continued through two decades. Many other investigators in this country and abroad have contributed to the search for the cause of these energy transformations following the ingestion of food. The early work was concerned with the varying amount of heat from the different individual

amino acids. Conflicting results were obtained, particularly with glutamic and aspartic acids, depending on the type of experiment and the nutritional state of the experimental animal. Extra heat production was compared with the nitrogen content of the amino acids, their glucogenic or ketogenic properties, their structural relation to hormones, and whether or not they were essential for growth, maintenance, or the formation of important cell constituents. Unexplained exceptions confronted each new theory. Lusk summarized the situation with the following statement: "The hypotheses which have been presented on specific dynamic action transcend one's power to coordinate them."

The development of the *in vitro* techniques for respiration studies of isolated tissues, cells, and enzyme preparations and more recently the use of isotope-labeled radicals in the intact animal as well as *in vitro* have opened the way to a broader understanding of energy transformations in intermediary metabolism. The tricarboxylic acid cycle, originally applied to carbohydrate oxidation, now appears to link carbohydrate, fat, and amino acids through a common two-carbon molecule, "active acetic acid." A new concept of the specific dynamic action of amino acids suggests that the metabolic breakdown of the amino acids in preparation for oxidation via the tricarboxylic acid cycle produces the waste heat. Transamination is proposed as the mechanism responsible for the variations in results on glutamic acid. Evidence is accumulating in favor of the energy-rich phosphate bond as a common intermediate essential link in energy transformations in living tissue. Despite these tremendous advances Rubner's dictum of "various possibilities" is still applicable.

Rubner visited this country in 1912 to attend the Fifteenth International Congress of Hygiene and Demography in Washington where he was honored as the international president of the Congress. One session was a notable symposium on specific dynamic action. The distinguished speakers were Rubner, Zuntz, Benedict, and Lusk. The following month (October 5, 1912) he delivered the Harvey Lecture in New

York on "Modern Steam Sterilization," thereby becoming an honorary member of the Society. He was elected a foreign associate of the National Academy of Sciences in 1924.

Rubner's friends have described him as a well-built man of striking presence, whose character was upright like his stature, searching for the truth with remarkable objectivity, a creative artist in research, a man of great proportions. A glimpse of Rubner's broad vision is found in his own words, "Mute and still, by night and by day, labor goes on in the workshops of life. Here an animal grows, there a plant, and the wonder of it all is not the less in the smallest being than in the largest."

WILLIAM H. CHAMBERS

LIVER STORAGE OF VITAMIN A BY MALE AND FEMALE RATS

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FOUR FIGURES

(Received for publication December 29, 1951)

In the most-used method for the bioassay of vitamin A the growth of rats on graded doses is taken as the criterion of potency. In another method the amount of vitamin A in the livers of dosed rats is taken as the criterion. Some workers who use the latter method grade doses in terms of body weight. Cross ('45) showed that grading is unnecessary for growth tests. Grading by weight now seems unnecessary for liver tests, but response depends on sex. For instance, Kimble ('39) and Callison and Knowles ('45) showed that female rats had more vitamin A in their livers than males. Ender ('34) found more vitamin A in the livers of cows than in those of bulls. Brenner, Brookes and Roberts ('42) found more vitamin A in the livers of female rats than in those of males after feeding liver oils. Esh and Sutton ('48) and Booth ('50) confirmed this with fish oils and carotene, but Lemley, Brown, Bird and Emmett ('47) observed no sex difference. Goodwin ('50) says that carotenoids are more abundant in the organs of females than in those of males in various species.

The difference in liver storage between the sexes may be caused by different growth rates or body weights, or may be connected more fundamentally with sexual dimorphism. This paper describes experiments designed to illuminate these hypotheses.

¹ Member of the scientific staff of the Agricultural Research Council.

METHODS

Dosing

Inbred piebald rats shortly after weaning at 21 days of age were caged individually and given a vitamin A-free diet of extracted casein² 17, sucrose 53, arachis oil 13, dried yeast 13, and salt mixture 4%. Each rat received 2 mg racemic α -tocopheryl acetate and 120 I.U. vitamin D₂ weekly. When growth ceased, dosing was begun. Carotene in arachis oil containing quinol, and halibut liver oil whether or not diluted, were given orally daily from a micrometer screw syringe. Portions of carrot or green leaf for a day's dose for a group of rats were weighed quickly, divided onto palettes, equalized by inspection, and given to individual animals. Portions were weighed concurrently for chemical assay of carotene.

Determination of vitamin A

Rats were killed with coal gas usually two days after the final dose. The livers and kidneys were removed, digested with 10% KOH and analysed (livers individually, kidneys usually pooled) for vitamin A by the method of Davies ('33) with such changes as extracting twice with ether. (See also Davies and Moore, '39.) Vitamin A (axerophthol) in the extract was determined with SbCl₃ photoelectrically.

Determination of carotene

Total carotene was assayed according to the technique of the Analytical Methods Committee ('50) and of Booth ('49): cold extraction of pigments by mixed light petroleum, acetone and quinol was followed by separation on an alumina-Na₂SO₄ mixture. To assess alimentary disappearance, carotene was estimated in the dose and in the feces collected from 5 to 70 hours after dosing. The "blank" pigment, previously excreted on the carotene-free diet, was subtracted from the fecal pigment (Booth, '47).

² Glaxo A/E or C/E.

Precision

Individual liver storage by rats of the same sex in the same group given the same dose varied widely. In general,

$$s = l^{0.74}$$

where l (liver) is the mean number of International Units of vitamin A found per liver for a group and s is the standard deviation of a single determination. Thus at low levels the scatter was high: the coefficient of variation rose rapidly for low mean storage values. Doses were therefore designed to give responses of over 50 I.U. per liver. The coefficients of variation for the two sexes were similar.

EXPERIMENTAL RESULTS

Liver deposit of vitamin A greater in females

After rats were given 4.45 mg of carotene in leaves during 17 days, the males had 290 and the females 450 I.U. vitamin A per liver. Other groups given 5.2 mg in carrots stored 315 and 470 I.U. In over 20 comparisons with carotene in oil and vegetables under various dosing conditions, more vitamin A was found in the livers of female rats than of males when both ingested the same quantity of carotene.

Intestinal absorption or digestion

Carotene is incompletely absorbed. If females absorb more than males, this might explain their greater storage. Judged by the disappearance of carotene during its passage through the alimentary tract, summarized in table 1, there was no sex difference.

Preformed vitamin A

Carotene is converted to axerophthol in the intestinal wall. To see if the sex difference in response was associated with efficiency of conversion, the process was bypassed by giving axerophthol in halibut liver oil. Liver analyses showed that females stored more than males even when given the preformed vitamin.

The effect of time on liver stores

Popper and Brenner ('42) gave fish oils to rats and observed that the liver vitamin A was lost by males sooner than by females. Bult and Sorgdrager ('38) had made a similar observation. That this sex difference held when carotene was used as source of the vitamin was shown by the following experiments.

A batch of rats, each having consumed the same amount of carrot, was divided into groups which were killed at intervals up to 21 days after the last dose. Liver stores in

TABLE 1
"Absorption" of carotene during alimentation

SOURCE OF CAROTENE	RATS PER GROUP		CAROTENE INGESTED	CAROTENE IN FECES		DIS- APPEARANCE	
	m	f		m	f	m	f
			μg	μg per rat		%	%
Green leaves	6	5	108	37.5	37.5	65	65
Green leaves	2	3	120	62	60	48	50
Oil	6	7	178	45	48	75	73
Oil	4	5	175	47	51.5	73	71
Carrot, cooked	5	5		various		45	42
Carrot juice	3	8		various		62	60
						Mean	61 60

males were lower than in females and both diminished at about the same rate: the liver-time curves for males and females were parallel, not divergent. When green leaves were fed as the source of carotene, there were similar sex differences but the diminution with time was slower in each sex.

Were the different levels in male and female livers due to immediate storage of different proportions of the dose, or to its being used up faster by males during the dosing period? If the amount stored immediately after one dose could be determined, then differential use during the interval between dosing and liver assay would be eliminated; i.e. the available surplus at zero time should be the same in both sexes. To

secure reliable response, the total dose must be large and spread over several days. Moreover, as shown below, single-dose response is not immediate. Hence the vitamin A deposited at once cannot be measured directly but has been estimated as follows. Experiments were grouped according to length of dosing period and the means plotted in figure 1. Extrapolation to zero dosing time gave a liver ratio value of 1.12. Thus males deposited less than females even from the same initial surplus.

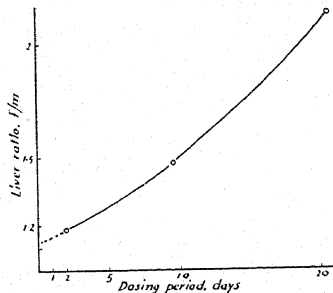


Figure 1

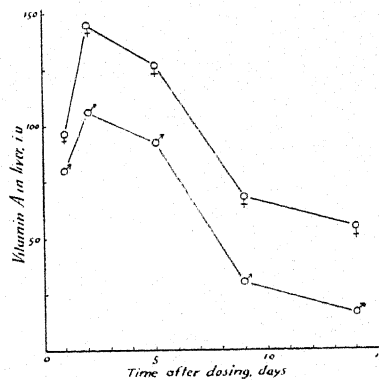


Figure 2

Fig. 1 The effect of dosing period on the female-to-male liver storage ratio of vitamin A. Each point represents the average of 8 or 9 experiments with a total of over 500 rats.

Fig. 2 Average liver stores of male and female rats, each given 500 I.U. of vitamin A at zero days.

With halibut liver oil a large amount of vitamin A can be given in one day, thereby partially obviating the complication of metabolism during the ingestion period. At 46 days of age 36 rats were each given 500 I.U. of vitamin A. Groups were killed at intervals. The results given in figure 2, show that maximum storage was reached only after two days and that the sex difference was observed initially. Another 36 rats from the same batch, each given 1,250 I.U. during two days, accumulated maximum storage only after 5 days. Another 70 rats were each given 640 I.U., with similar results.

It is concluded that liver stores are less in males than in females from the start, that both lose their stores at about the same rate (International Units per rat per day), and hence that the ratio (f/m) increases with time. This explains why the ratio increases with length of dosing period.

Relation with growth

Since vitamin A is essential for growth, faster-growing rats might use more of the vitamin, and therefore have less surplus for storage than slower-growing rats. Growth rate and storage might then be inversely related. Males grow faster than females: the mean of the ratios — male growth during the dosing period divided by female growth — in 24 groups comprising 217 rats in this study was 1.72, and the coefficient of variation of the 24 ratios was only 5.6. High and Day ('51) concluded that the sex liver difference was due chiefly to differences in growth. Correlation between growth and storage was therefore sought as follows.

Within a group of rats of one sex receiving the same dose, growth rates varied from rat to rat. The root mean square of the coefficients of variation for the rates in 26 groups of males and 27 groups of females was 18.3. The individual liver storage of vitamin A also varied. The 258 rats were used to calculate the correlation between growth rate and storage level within these 53 groups. This was — 0.02 for males, — 0.08 for females and — 0.05 for all rats. In other words, about the same percentage of a given dose was stored by faster-growing rats as by slower-growing rats of the same sex. These correlations, which are not significant ($P = 0.05$), make it improbable that greater growth rate was the cause of males' storing less surplus vitamin A.

As is shown below (table 2), older rats stored less of a given intake than younger rats which were growing faster. This observation also confutes the growth hypothesis.

Relation with body weight

More surplus might be stored by small animals than by larger animals from a given intake. Male rats are normally bigger than females and thus, if weight affects storage, would store less. The finding by Cross ('45), of no correlation between weight and growth on dosing A-free rats with carotene, is against that possibility. The body weights of 166 males and 191 females in certain of the present experiments, midway through the dosing period, were recorded. Within any isogestic group of the same age and sex the individual weights varied from rat to rat. The vitamin A found per liver also varied. The correlation for males was

TABLE 2

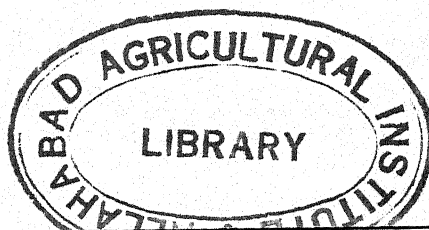
Effect of age or body weight on liver storage of vitamin A from carotene ingested

CAROTENE IN	AGE ¹	WEIGHT ^{1,2}	DOSE	LIVER A ²
	<i>days</i>	<i>gm</i>	<i>mg</i>	<i>I.U.</i>
Oil	62	120	1.74	224
	91	144	1.54	105
Leaves	62	116	1.42	200
	108	154	4.00	220

¹ Half-way through the dosing period of 22 days.² Mean for males and females.

— 0.17 ($P=0.03$), for females 0.02, and for both sexes — 0.09 ($P=0.11$). This hint that large males stored less than small males is not decisive enough to account for the different storage by males and females.

Seemingly the problem of whether body size controls storage could be solved by comparing rats of different ages and therefore different weights. However, the question arises, how much vitamin A must rats have for health between depletion and the beginning of the delayed test? The difficulty was suppressed rather than solved by using large doses. Young rats of both sexes were used at depletion to compare with older rats which had received barely enough of the vitamin for maintenance. Table 2 shows that twice



as much carotene was needed by larger as by smaller rats for similar liver storage. However, these results do not prove that body size is the dominating factor, because the larger rats were older than the smaller ones. It was not clear whether age or weight determined the storage level. There is evidence from other experiments that the absorption of vitamin A is impaired by vitamin A deficiency. The older rats had been deficient a long time.

Storage by very young rats

The difference in size between young males and females is negligible. Weanling rats were divided into groups and given a large dose each of carrot exceptionally rich in carotene (590 p.p.m.). Other weanlings were given halibut oil. The males weighed 38 and the females 37 gm. The results of 5 comparisons showed that males stored about the same as females. This supports the body weight hypothesis. But it is arguable that such immature rats do not yet show every sex dimorphism.

As rats grow the male/female weight ratio increases. Results of 23 experiments showed no correlation between body weight ratios and liver storage ratios for the sexes. Were weight the determining factor in liver storage, a positive correlation would be expected.

The effect of age

An attempt was made to separate the effects of age and weight as follows. Female rats were given a vitamin A-free diet supplemented with leaves and carrots supplying 32 μ g of carotene per rat weekly — enough vitamin A for maintenance but not for liver storage. At 37 weeks of age the rats had almost finished growing. Ten were given two doses of halibut oil (1,240 I.U., total) and after 14 days their livers and kidneys contained 414 ± 22.3^3 and 5 I.U. vitamin A, respectively. Their sisters were kept on the diet until they were

³ Standard error of the mean.

39 weeks older but only 20 gm heavier. There were occasional signs of deficiency and three rats died. The 11 survivors were given two doses of halibut oil (1,575 I.U.) and 14 days later their livers and kidneys were found to contain 519 ± 23.6 and 13 I.U. Thus the older rats stored vitamin A as efficiently as the younger ones, 34% being recovered in each group. In another experiment, differing as to times, doses, and so forth, the same conclusion was reached.

If efficiency of storage is unaffected by age, the results in table 2 are best explained by the inverse weight hypothesis.

TABLE 3

The effect of litter size on liver storage and weight of rats
(Each rat was given 510 I.U. vitamin A in halibut liver oil)

Rats per litter	Two		Twelve	
Sex	m	f	m	f
Rats per group	3	6	9	8
Weight per rat, gm ¹	138	115	78	74
S.e., gm		5.0	4.4	1.5
Growth rate ²	48	43	38	34
I.U. per liver	78	99	73	95
S.e., I.U. per liver		16.7	11.7	11.3

¹ Before killing at average age of 43 days.

² Mean increase in grams in 13 days prior to sacrifice.

The effect of underdevelopment on liver storage

Weanling body weights vary with litter size because the mother's milk must be shared. In each of 6 litters all the young except one male and one female were killed at birth. Two other litters were not so reduced and comprised 6 males and 6 females each. All were weaned as usual to the vitamin A-free diet. At 40 days of age, when twins weighed nearly twice as much as duodecets, rats from each litter were given 510 I.U. of vitamin A in halibut oil in two days. On the third day the rats were weighed, killed, and the livers analysed. The large females in twins stored more vitamin A than the *smaller* males in duodecets. Table 3 shows that the weights

of the rats, as controlled by litter size, had no effect on the storage by males or by females. The weight difference between the sexes was lower for duodecets than for twins, as might be expected from the observation of Jones ('51), but the liver ratios were the same for each litter type. Results with litters of 8 — e.g., males weighing 103 gm stored 77 I.U. per liver — fitted into the scheme but are omitted from the table. The difference between litters cannot have been caused by greater liver reserves in twins, for there was negligible vitamin A in the livers of 15 undosed rats; and in another experiment female twins given the A-free diet had, at 43 days of age, only 9 I.U. per liver against 2 I.U. in duodecet male livers. The female twins were growing faster than the male duodecets. Hence the low storage of the duodecet males cannot have been due to fast growth.

Growth on a limited regular intake of vitamin A

During bioassay by growth the dose of vitamin A given to rats is designedly too small for maximum growth. On this small dose, males grow faster than females. In 12 widely separated tests on different materials the average growth ratio was 1.43 (s.d. = ± 0.182). The faster growth of males suggests that their need of the vitamin for growth is no greater than that of females.

Growth and survival on limited single doses

Mayer and Krehl's ('48) male rats showed deficiency symptoms sooner than females. But when rats in this laboratory were given the vitamin A-free diet prior to dosing in 8 bioassays in which cessation of increasing body weight was the criterion of vitamin A deficiency, females "ran out" only about one day later than males. This was surprising because (fig. 2), after ingesting similar amounts of vitamin A, males lost their liver stores sooner than females. Perhaps young males have, at weaning, larger reserves than females. The results of liver tests on 7 groups of about 13 rats each,

averaging 38 days of age, on the vitamin A-free diet refute this explanation: males had 9.1 and females 7.6 I.U. — a difference of only 1.5.

Storage in kidney

That male rats lose their liver stores of vitamin A sooner than females, yet survive equally, suggests a reserve elsewhere which is greater in males than in females. Most of the stores are usually assumed to be in the liver, with small amounts in the kidney. Johnson and Baumann ('47) and Moore and Eden ('51) found more vitamin A in kidneys than in livers of rats when the total was small. Johnson and Baumann ('48) found that kidney stores rose as liver stores fell in rats on a deficient diet. Sex differentiation in kidney storage was therefore studied. Meanwhile Moore and Sharman ('50) reported more vitamin A in male than in female kidneys after dosing rats for a month. This was confirmed using single doses (Booth, '50) and has been amplified as follows.

Male rats on the A-free diet at 33 days of age had 5.5 and females had 4.7 I.U. per kidney pair. At 41 days of age males had 1.3 and females 2.0 I.U. Thus sex difference on this diet was negligible.

Similar rats were each given 500 or 1,250 I.U. of vitamin A in halibut oil and killed two days later. The kidneys of males and of females contained 10 I.U. of vitamin A per pair. In contrast to liver, the amount of vitamin A stored in the kidneys was scarcely affected by increasing the dose two and one-half times. Other experiments confirmed both conclusions, but when assayed more than two days after dosing more vitamin A was found in male than in female kidneys. This is seen in figure 3, drawn from the pooled results of several experiments in which 238 vitamin A-free rats were given 480 to 1,250 I.U. of vitamin A in halibut oil, divided into groups and sacrificed at intervals. Male kidney levels rose with time, as stated by Johnson and Baumann ('48) for "rats" without mention of sex, but in females the rise was less marked.

Kidneys were usually pooled, but in one experiment they were analysed in pairs. The standard deviation was independent of the level of response and had a pooled value of 3.53 I.U. for the 5 groups of 7 males and 5 groups of 7 females. Hence the standard error was 1.34, which indicates the precision of figure 3.

The sex difference in kidney storage cannot account for differential liver storage because the kidney difference is too

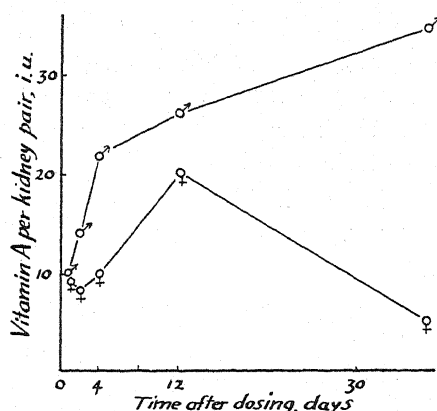


Fig. 3 Effect of time on kidney stores of vitamin A in rats given halibut liver oil on zero day.

small and the liver difference anticipates the kidney difference. Both differences may be seen by comparing figures 2 and 3.

Responses by males and females to graded doses

Rats were given graded doses of halibut oil during 9 days and killed two days later. Several conclusions are drawn from the results of 64 liver analyses summarized in figure 4. (1) Rats given lower doses, though not represented in the figure because their liver responses were zero, nevertheless grew satisfactorily. (2) The dose had a threshold value below which no vitamin was found in livers. This value depends on length of dosing period. (3) The threshold value was higher

for males than for females. In this experiment the extrapolated values were 340 and 270 I.U. (4) The response for each sex was almost linear over a 60-fold dose range. (5) The extrapolated male and female curves met at zero dose value but below zero liver response. (6) The difference between male and female storage was not constant: the response curves had different slopes. The difference was proportional to the dose,

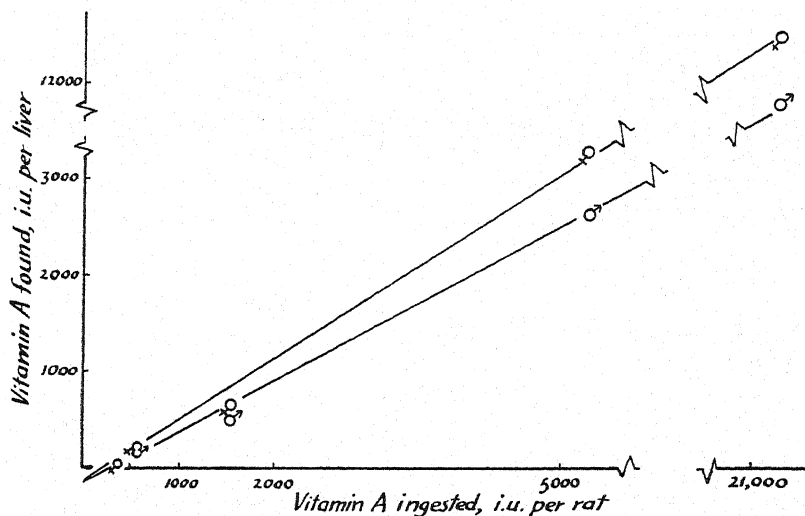


Fig. 4 Dose-response curves for rats given halibut oil over 9 days. The scales have been divided by three for the higher points.

of which under these conditions (9-day dosing period) it was 11%. In other words, 11% more of a given dose disappeared in the male than in the female.

It follows from items 3 and 4 that

$$l = k(i - t),$$

where l is the vitamin A found in the liver, i is that ingested and t is the threshold. In these experiments $k = 0.53$ for males and 0.63 for females.

More was ingested by males than by females for a given storage level. The percentage difference varied a little with level but near the middle of figure 4 it was 24%. Males weighed

23% more than females. The correspondence of the two differences supports the body weight hypothesis. If faster growth caused greater disappearance in males, a constant difference would be expected in the liver responses, independent of dose, whereas a proportionate difference was found.

Castration

Bult and Sorgdrager's ('40) castrated rats lost their liver stores of vitamin A at rates intermediate between those for males and females. The present author has studied castration as follows. Male rats were castrated and females were ovariectomized when 32 days old. At 46 days of age they, and normal litter mates, were given halibut oil. After 2, 26 and 46 days rats from each group were killed. Castrated males had slightly more liver vitamin than normal males and lost their stores very slightly more slowly; i.e., castration tended to induce femininity. The ovariectomized females stored more vitamin A than normal females, lost their stores very slightly more slowly and grew faster. In growth rate they resembled males, but in vitamin A storage the small difference from normal females was away from the direction of the males. Castrated males on each date had kidney vitamin A values between those of males and females. Ovariectomized females also stood between males and females, closer to females.

CONCLUSIONS

Items 5, 8, 9 and 11 in the summary disprove the growth rate hypothesis for differential liver storage between the sexes. Moreover, different growth rates could hardly account for the difference found immediately after dosing.

Another hypothesis, that liver storage is inversely related to body weight and males therefore store less vitamin A in their livers because they are larger than females, is supported by summary items 7 and 11. Items 6 and 8 and the lack of correlation between weight ratios and liver ratios, however, provide evidence against the hypothesis. The logic

of these deductions is explained in the relevant sections under Experimental Results.

The observations that (1) sub-threshold doses induced no liver storage, (2) rats given sub-threshold doses grew normally, (3) sex difference in storage was proportional to dose, and (4) only half the vitamin ingested appeared in the liver, suggest that vitamin A is also stored elsewhere than in the liver, and in greater amount in males than in females. The alternative site cannot be the kidney, for the amount there was small and did not rise with increased dose. No other tissue is reputed to contain appreciable amounts. Possibly the vitamin which disappears is destroyed: this hypothesis, however, fails to explain how rats survive after their liver stores are depleted. Evidence accumulates for the existence of a form of vitamin A not detected by recognized chemical tests (Kaunitz and Slanetz, '50; Le Gallic, '47). Readier storage of this vitamin by males than by females would explain the survival anomaly.

Thus it is not known what happens to the unrecovered proportion of the ingested vitamin. Nor is the problem of unequal disappearance in the sexes solved, but it is hoped that light has been thrown on it. Preliminary communications show that several workers are studying the effects of hormones on vitamin A metabolism. Their results may clarify the problem. Another approach might be to use species in which females are as large as males. It would be interesting too to know whether other axerophthoids are stored unequally by the sexes.

Whatever is the explanation of differential storage, two practical conclusions can be stated; namely, that in bioassaying by the liver storage method, (1) doses should not be graded according to individual body weights, although males may be given larger doses than females, and (2) data for males and females must receive separate arithmetical treatment.

SUMMARY

1. Vitamin A-free rats were given carotene in oil or vegetables, or halibut liver oil, then killed. Vitamin A was assayed by the SbCl_3 test in the livers or kidneys or both.

2. The standard deviation of a liver determination of vitamin A within any one group varied with the 0.74th power of the mean liver value for the group.

3. After they had ingested carotene, more vitamin A was found in the livers of females than of males. Both lost their stores at similar rates. Hence male livers depleted sooner than female and the female/male storage ratio increased with time.

4. The absorption efficiency of carotene was not linked to sex.

5. Neither sex showed correlation between storage and growth rate.

6. Correlation was negligible between storage and body weight among rats of one sex at the same age similarly dosed.

7. Infant rats had negligible sex differences in body weight and liver storage. Young rats stored more than middle-aged rats from the same intake, but old rats stored the same proportion as middle-aged rats of similar size.

8. Twin females, though weighing more and growing faster than duodecet males, stored more vitamin A.

9. On vitamin intakes too low for maximum growth, males outgrew females. After being given a vitamin A-free diet from weaning, or after one dose of vitamin A, males showed deficiency symptoms only slightly sooner than females.

10. Males and females had similar kidney levels shortly after a dose. The level in male kidneys increased with time, but in female kidneys no comparable rise was found.

11. Male and female dose-response curves had different slopes. The difference between male and female liver stores at different doses was a constant proportion (11%) of the dose. The threshold dose, below which there was no storage, was higher for males than for females.

12. Items 5, 8, 9 and 11 show that the sex-liver-storage difference cannot be related to different growth rates. Another hypothesis, that liver storage is inversely controlled by body weight, is supported by items 7 and 11, refuted by 6 and 8. This and other possibilities are discussed under Conclusions.

ACKNOWLEDGMENTS

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NUTRITIONAL STUDIES OF THE CHINCHILLA, WITH SPECIAL REFERENCE TO ASCORBIC ACID AND THIAMINE

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INTRODUCTION

No studies of the nutrition of the chinchilla, *Chinchilla brevicaudata* or *Chinchilla laniger*, are available in the literature. The basic procedures used here were adapted from those employed previously by Claussen and Clark ('43), Cooperman et al. ('43), Hamilton and Hogan ('42), and Hogan and Hamilton ('44) in studying the vitamin requirements of other rodents.

The present status of the chinchilla industry places two rather severe restrictions on the use of these animals for experimental purposes. First, the cost of the animals themselves (approximately \$1,000 per breeding pair) limits the number available. Second, the necessity on the part of commercial breeders to keep their females for breeding stock almost eliminates the use of females in experiments where mortality is likely to be high.

Because of the fact that the experiments reported here employed limited numbers of animals, the conclusions drawn are considered tentative, pending further work with larger groups of animals and perhaps several changes in the purified diet.

EXPERIMENTAL

Management of animals

The chinchilla is a strictly herbivorous rodent native to South America. Bickel ('31) and Prell ('34) have made ana-

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tomical studies of the chinchilla. Pierce ('44) and the U. S. Fish and Wildlife Service ('44) have described standard practices in herd management.

In the present investigations the animals were housed in wire cages ($\frac{1}{2}$ " mesh) $18'' \times 18'' \times 18''$, to the outside of which was connected by means of an open door, $8'' \times 9''$, a sheet metal "nest box," $11'' \times 11'' \times 8''$. The nest box also had a wire bottom. Each animal was separated from that below it by a sheet metal dropping board. Water was given ad libitum from inverted bottles. The temperature was maintained between 50° and 60°F . The animals were allowed to dust themselves in clean Fuller's earth for 5 to 10 minutes daily.

Procedure, Part I

In preliminary experiments using mature animals, a variety of purified diets were completely rejected. Starch, dextrins, and sucrose as carbohydrate sources, the addition of steam distillates from alfalfa and clover as flavoring agents, and the addition of monosodium glutamate as a flavor intensifier all failed to improve acceptance. Purified diets in the form of mashes, pellets, and moistened (dustless) powders were also rejected by mature animals.

On the basis of preliminary experiments with weanlings, a purified diet (ration 1, table 1) was prepared with the vitamin concentrations expressed in terms of milligrams or micrograms per 15 gm ration, since the animals eat an average of 15 gm of ration per day during the growth phase studied.

Ten animals received from commercial breeders the day of weaning (age, 5 to 11 weeks) were divided into three groups. In all experiments reported here the animals were fed ad libitum. Group 1, consisting of two animals fed a "ranch-type" ration of rabbit pellets and a fermentation product of alfalfa meal,² served as controls. Group 2 con-

²Enzymalfa, Jeffries Laboratories, Roanoke, Virginia; a product prepared by fermentation of alfalfa meal and sold as a supplementary feed for poultry. "Wayne" Rabbit Pellets.

sisted of 4 animals fed ration 1. Group 3 consisted of 4 animals fed ration 1 plus 2 mg ascorbic acid in a sucrose solution fed orally by pipette each day. The selection of animals for groups 2 and 3 was made on the basis of their

TABLE 1
Composition of purified diets used

CONSTITUENTS	RATION	
	1	2
Sucrose ¹ (%)	48	45
Casein (%)	25	25
Cellulose (%)	17	20
Vegetable fat (%)	5	5
Ashed alfalfa (Roine et al., '49; %)	2.5	2.5
Salt mixture (U.S.P. XII, no. 2; %)	2.5	2.5
Biotin ($\mu\text{g}/15\text{ gm}$)	2.0	4.0
Vitamin B ₁₂ ($\mu\text{g}/15\text{ gm}$)	0.5	1.0
Thiamine hydrochloride (mg/15 gm)	0.2	0.4
Riboflavin (mg/15 gm)	0.3	0.6
Pyridoxine hydrochloride (mg/15 gm)	0.2	0.4
Calcium pantothenate (mg/15 gm)	0.3	0.6
Niacin (mg/15 gm)	0.7	1.4
<i>p</i> -Aminobenzoic acid (mg/15 gm)	14.0	28.0
<i>i</i> -Inositol (mg/15 gm)	35.0	70.0
Folic acid (mg/15 gm)	0.07	0.14
Choline chloride (mg/15 gm)	15.0	30.0
Menadione (mg/15 gm)	0.5	1.0
Alpha-tocopherol acetate (mg/15 gm)	30.0	60.0
Vitamin A (I.U./15 gm)	50.0	100.0
Vitamin D (I.U./15 gm)	10.0	20.0
MnSO ₄ ·4H ₂ O (mg/15 gm)	3.05	3.05
ZnSO ₄ ·7H ₂ O (mg/15 gm)	3.05	3.05
CuSO ₄ ·5H ₂ O (mg/15 gm)	0.6	0.6
CoCl ₂ (mg/15 gm)	0.5	0.5
KI (mg/15 gm)	0.6	0.6

¹ Sucrose supplied as Domino cane sugar; casein as "vitamin-test," General Biochemicals, Inc.; cellulose as "non-nutritive diet," General Biochemicals, Inc.; vegetable fat as Crisco; and vitamin B₁₂ as experimental animal protein factor, Merck and Co., Inc.

willingness to accept the sucrose solution voluntarily because it was found that force-feeding made the animals extremely nervous. The animals were weighed weekly for 15 weeks.

TABLE 2
Growth rates of individual chinchillas with and without vitamin C
 (grams per week)

ANIMAL NO.	GROUP 1		GROUP 2				GROUP 3			
	1	2	3	4	5	6	7	8	9	10
Initial weight (gm)	175	190	135	280	230	175	205	225	130	160
Weeks 1-7	(Ranch diet)			(Ration 1)			(Ration 1 plus 2 mg ascorbic acid per day) ¹			
	14	14	14	— 2	— 3	6	11	16	Died	11
Weeks 8-11	(Ranch diet)		(Ration 1 plus 2 mg ascorbic acid per day) ²				(Ration 1 plus 2 mg ascorbic acid per day) ²			
	4	7	5	— 1	7	— 1	18	10	..	9
Weeks 12-15	(Ranch diet)		(Ration 1, no ascorbic acid, other vitamins doubled)				(Ration 1, no ascorbic acid, other vitamins doubled)			
	16	9	8	9	15	8	18	12	..	9
Average gain/week (gm)	10.6		5.8			12.5				

¹ In sucrose solution given orally by pipette.

² Mixed into the dry ration each day just prior to feeding.

During the 8th through 11th weeks the procedure was altered in that 2 mg ascorbic acid/15 gm ration were mixed into the feed of all animals on purified diets (but not that of the controls) each day just prior to feeding.

During the 12th through 15th weeks all animals in groups 2 and 3 were offered the same purified diet except that the concentrations of all the vitamins with the exception of ascorbic acid were doubled. No ascorbic acid was fed to either group 2 or group 3. The results in terms of growth rates are shown in table 2.

Procedure, Part II

Ten animals received from commercial breeders the day of weaning (age 5 to 8 weeks) were divided into 4 groups. All animals were offered ration 2, table 1, without thiamine for one week. From the second week on, group 1 (two animals) continued throughout the experiment on the thiamine-free diet. Group 2 (three animals) was fed the same purified diet with 0.05 mg thiamine/15 gm ration. Group 3 (three animals) was fed the same diet with 0.10 mg thiamine/15 gm ration. Group 4 (two animals) was fed the same diet with 0.40 mg thiamine/15 gm ration. All animals were weighed weekly for 7 weeks. The results are shown in table 3.

DISCUSSION OF RESULTS

Part I

No data on the normal growth of animals from the same stock as the experimental animals is available outside of the two used as controls in this experiment. It is therefore impossible to state whether the growth attained (10 to 12 gm/week) is typical of the healthy animal under ranching conditions. Data from other stock (National Chinchilla Breeders of America, '49) show an average growth rate over a comparable age span of 17 gm/week, but the number of animals is not given and the individual rates ranged from 8.7 to 24.8 gm/week. Because only small groups of animals were

used here, it can only be concluded that the growth of weanlings on the modified diet appears to have been nearly normal.

At the end of the 7th week the poor growth response of the animals in group 2 (without vitamin C) appeared to indicate that vitamin C is a dietary essential for the growing chinchilla. The possibility remained, however, that the selection of animals for groups 2 and 3 was biased. Should

TABLE 3
Weight responses of weanling chinchillas to various levels of thiamine

	GROUP 1, RATION 2 WITHOUT B ₁		GROUP 2, RATION 2 WITH 0.05 MG B ₁ /15 GM			GROUP 3, RATION 2 WITH 0.1 MG B ₁ /15 GM			GROUP 4, RATION 2 WITH 0.4 MG B ₁ /15 GM	
Animal no.	1	2	3	4	5	6	7	8	9	10
Initial wt. (gm)	230	250	210	210	210	220	190	155	185	170
Net gain or loss in wt.	(gm)									
Weeks after depletion										
1	5	—15	0	5	—5	—5	—10	10	15	15
2	10	—5	5	0	5	—5	—10	30	40	20
3	15	—5	35	15	0	25	—10	45	25	30
4	25	0	35	15	0	30	10	45	55	60
5	25	—5	20	25	0	40	10	60	65	60
6	35	—10	50	25	0	50	15	55	65	70
Ave. net gain (gm)	12.5		25			40			67.5	
Ave. gain/week (gm)	2.1		4.1			6.7			11.3	

the poor growth of group 2 have been due to a deficiency of vitamin C, then the addition of the vitamin to the ration, as was done in the second phase of the experiment, would have been expected to result in improved growth. This was not found to be the case.

Since the difference in the growth rates of the two groups did not appear to be the result of a vitamin C deficiency, it was thought that perhaps one of the other vitamins was present in the ration at a slightly submarginal level. Under

such conditions, those animals possessing a cecum microflora that was active in the synthesis of the deficient vitamin might show a normal growth response, while those with a slightly less active flora might show impaired growth. The fact that one animal in group 2 grew as well as either the controls or the average of group 3 supports the conclusion that some factor other than vitamin C was causing the difference between groups 2 and 3. The possibility also existed that the chinchilla requires some unknown vitamin for growth not present in the purified diet, which was being supplied to some of the animals by bacterial synthesis and not to others.

In the third phase of the experiment, where vitamin C was omitted from the diets of both groups and the amounts of all other vitamins were doubled, the animals of group 2 showed a marked increase in growth rate, while those in group 3 continued to grow at about the same rate as before. This growth in group 2 was in contrast to the failure of the animals to show appreciable growth during the preceding 6 weeks. The apparent growth of animal 5 during the 8th to 11th weeks is attributed to the fact that he became constipated during the 6th week, stopped eating, and lost weight badly. Recovery came during the 8th week, without change in diet or handling, and is reflected in the increased weight shown between the 8th and 11th weeks.

This evidence indicates that vitamin C is not a dietary essential for the growing chinchilla.

Part II

Animals receiving no dietary thiamine showed a decided tendency to develop alopecia on the rear regions of the body, including the hind legs down to the toes. Animal 6 (group 3) first showed the alopecia during the depletion week, but began to grow new fur about one week after thiamine was included in the diet. Both animals in group 1 (no thiamine) began to show alopecia during the 4th week and failed to grow new fur. Beginning during the 4th week both animals in group 1 also showed signs of impaired co-

ordination when rolling in their dust pans. By the end of the 7th week neither animal was able to roll over completely without a great deal of struggling.

The probable error of the data relating to group 4 is 2.38, making that group significantly different from group 3 at a 1% level. The data indicate that dietary thiamine is essential for the growing chinchilla. The rate of growth attained by group 4 was of the same order of magnitude as that obtained in the preceding experiment by either the control group or group 3 (10 to 12 gm per week). Since the animals ate an average of 15 gm of ration per day, it would appear that the minimum daily requirement of the growing chinchilla for thiamine probably lies somewhere between 0.1 and 0.4 mg.

Increasing the cellulose to 20%, as in ration 2, table 1, resulted in more normal size and texture of the feces. Ration 2 is therefore considered an improvement over ration 1.

SUMMARY

1. The development of a purified diet for chinchilla weanlings which apparently will support normal or nearly normal growth for 15 weeks is reported. The diet is similar to those used for other rodents except that it contains 20% roughage, lower levels resulting in constipation.

2. Based on the use of such a diet, evidence is presented indicating that vitamin C is not a dietary essential for the growing chinchilla.

3. The minimum daily thiamine requirement of weanling chinchillas for growth appears to be between 0.1 and 0.4 mg.

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STUDIES ON ESSENTIAL FATTY ACID DEFICIENCY IN THREE STRAINS OF MICE¹

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TWO FIGURES

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Although essential fatty acid deficiency has been studied in detail in the rat, very little work has been done with the mouse. White et al. ('43) described the syndrome produced with a fat-free diet in albino mice of their own laboratory strain. They found that the deficiency could be cured or prevented by the addition of lard to the diet. In the present paper we report our observations on the fatty acid deficiency syndrome in three highly inbred strains of mice. While this work was in progress, Decker et al. ('50) reported that they had been able to produce a chronic deficiency in mature mice.

EXPERIMENTAL

Mice⁴ of the C57 black, dba, and C3H strains were used. The composition of the diets is given in table 1. Each mouse strain was set up in 4 groups (table 2). Group I was a negative control group maintained on the deficient diet, P-54.

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⁴ The C3H and dba mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The C57 black mice were bred in our own stock colony.

Group II consisted of positive controls receiving the complete diet, P-53. Group III was supplemented with varying levels of methyl linoleate after development of the syndrome. Group IV was supplemented with 5 mg of methyl linoleate per day at the time the negative controls were placed on diet P-54.

The mice were started on the experiment when they weighed between 12 and 16 gm. Those receiving diet P-54 were allowed to develop the deficiency symptoms. When they reached this stage, which was accompanied by a loss of weight, a

TABLE 1
The composition of the basal diets

CONSTITUENTS	FAT-FREE (P-54)	CONTROL (P-53)	VITAMIN SUPPLEMENT PER KG OF DIET	
	%	%		
Casein, vitamin-free ¹	25	25	Thiamine hydrochloride	5 mg
Sucrose	68	53	Riboflavin	5 mg
Salt mixture ²	5	5	Pyridoxine hydrochloride	5 mg
Ruffex	2	5	Biotin	100 mg
			Calcium pantothenate	30 mg
Lard		5	Choline chloride	500 mg
Hydrogenated vegetable fat ³		10	Vitamin A ⁴	67,500 I.U.
			Vitamin D ⁵	5,000 I.U.
			α -Tocopherol acetate	200 mg
			Folic acid ⁶	5 mg

¹ Labco.

² Osborne and Mendel.

³ Crisco.

⁴ Given in the form of a concentrate (1,000,000 I.U./gm) supplied by the Nopco Chemical Co., Harrison, N. J.

⁵ Given in the form of Drisdol (Winthrop-Stearns Inc., New York, N. Y.).

⁶ Kindly supplied by the Lederle Laboratories Division, American Cyanamid Co., through the courtesy of Dr. T. H. Jukes.

TABLE 2
Number of mice on various dietary regimens

STRAIN	GROUP			
	I	II	III	IV
C57	8	18	22	9
dba	14	4	10	4
C3H	13	4	5	4

certain number of the animals were given 2, 5 or 10 mg methyl linoleate per day. In the case of the C57 and dba mice, the development of the neck lesion was used as the criterion in determining when to start the treatment. In the case of the C3H mice, a significant drop in weight was the determining factor. A cure was considered to have taken place when the external signs of the deficiency disappeared and the animals had gained weight.

The effect of pre-depletion of the weanling on the time required for the development of the syndrome was determined by depletion of the mother during the latter part of lactation.

The mice were weighed on alternate days and were given the indicated supplements daily. The alcohol solution of methyl linoleate was given by spreading the solution through the top layer of food in the feed cup. Water was supplied *ad libitum*.

RESULTS

Most of the symptoms noted with the three strains were the same as those found previously by White et al. ('43). These included a dermatitis of the skin and the extremities, scaliness of the ears, alopecia, and retardation of growth. A neck lesion, characterized by an initial alopecia of the neck followed by a dermatitis and lesioning, was also shown by all three strains. As is shown in table 3, in which data obtained on groups I and III have been included, most of the mice developed a "browning," which was a lightening in fur pigmentation in the lower dorsal region.⁵ In two-thirds of the dba mice a "spectacle eye" condition was observed. This symptom was also noted occasionally in the C57 mice.

It was also found that pre-depletion of the C57 weanling resulted in development of the syndrome in less than one-half the time required with mice weaned on a stock diet containing all the nutrients. The effect of pre-depletion on the dba mice was even more marked; they developed the visible

⁵ Preliminary studies by Miss Cecilia Torda in our laboratory indicate that a similar "browning" occurs in black rats (Evans-Long strain) kept on the deficient diet, P-54.

symptoms in less than one-third of the time required by mice weaned on a stock diet.

The daily requirement for methyl linoleate was found to be approximately 5 mg. As can be seen in table 4, 2 mg per day were totally inadequate for the cure of the syndrome.

TABLE 3

Average day of development of symptoms in fatty acid-deficient mice

SYMPTOMS	C57 (11) ¹ (Stock) ²	C57 (19) ¹ (P-54) ²	dba (15) ¹ (Stock) ²	dba (9) ¹ (P-54) ²	C3H (18) ¹ (Stock) ²
Dandruff	33 (11) ³	10 (19) ³	30 (2) ³	9 (1) ³	30 (14) ³
Browning	47 (10)	22 (18)	50 (14)	..	50 (13)
Neck lesion	71 (9)	45 (18)	41 (10)	12 (6)	44 (11)
Alopecia	76 (8)	41 (14)	51 (9)	16 (6)	66 (9)
Ear thickening	103 (6)	40 (17)	43 (10)	11 (5)	66 (9)
Skin dermatitis	107 (6)	55 (9)	57 (7)	12 (8)	..
Tail dermatitis	113 (4)	56 (6)	56 (6)	40 (1)	55 (14)
"Spectacle eye"	46 (9)	11 (6)	..

¹ Figures in parentheses represent number of mice used.

² Diet on which the mice were weaned.

³ Figures in parentheses represent number of mice which developed symptoms.

TABLE 4

Comparison of curative effects of varying amounts of methyl linoleate in the three strains

SUPPLEMENT PER DAY	C57		dba		C3H	
	No. of mice	No. of cures	No. of mice	No. of cures	No. of mice	No. of cures
mg						
2	11	0	3	0
5	9	8	5	5	5	4
10	2	2	2	2

Preliminary experiments indicated that a 3-mg level of methyl linoleate produced only a temporary, partial remission of the syndrome.

When the mice were given 5 mg of methyl linoleate per day before the development of the symptoms, they maintained normal growth at a slightly lower level than the positive controls and did not show any external symptoms of the deficiency.

The growth of these mice as compared with that of the positive controls is shown in figure 1 for C57 mice. Results with the dba and C3H mice were the same as those with C57 mice.

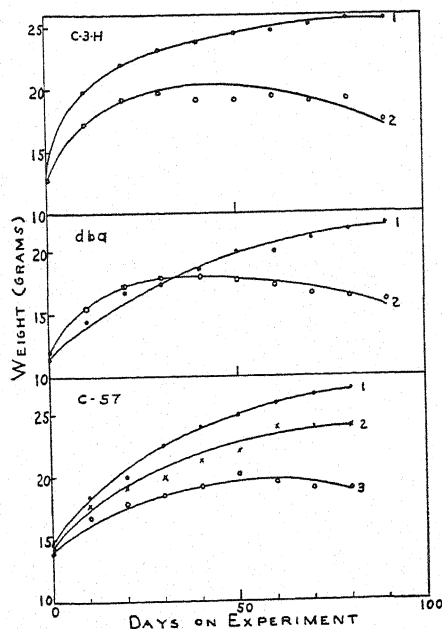


Fig. 1 Comparison of average growth rates for mice of the three strains receiving diet P-53 (curves marked 1), and for those receiving the deficient diet P-54 (curves marked 2 for the C3H and dba animals, and curve 3 for the C57 mice). The average growth rate for 4 mice of the C57 strain which were given 5 mg methyl linoleate per day prior to the development of the symptoms is also shown (curve 2).

In figure 1 are shown the curves of normal and deficient mice. Figure 2 shows the effect of supplementation with 5 mg of methyl linoleate per day on the growth of deficient mice.

In the case of 4 dba mice in which supplementation with 5 mg of methyl linoleate was discontinued after the cure of the deficiency, a loss in weight was noted, and three of these mice died before the syndrome appeared. The 4th mouse again showed the external symptoms of the deficiency before death.

Mice of the dba strain were found to be extremely sensitive to the essential fatty acid deficiency. As may be seen in table 3, this strain developed the syndrome more rapidly than either the C57 or C3H mice. In general, there was a high rate of mortality among the dba mice in the early stages of the experiment; about one-third of these mice died before the syndrome could develop.

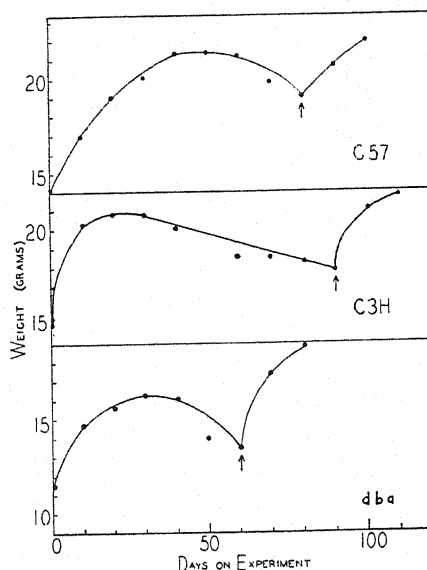


Fig. 2 Curves showing the effects of supplementation with 5 mg of methyl linoleate per day in deficient mice of the three strains. The arrows indicate the time when supplementation was begun.

Apparently there is some storage of the essential fatty acids in mice weaned on a stock diet, for it was found, in general, that the earlier the mice were started on the experiment after birth, the earlier did their symptoms develop.

Since mice which were supplemented with 5 mg of methyl linoleate per day, prior to the appearance of the external symptoms, developed normally, and since this quantity of the ester given per day was sufficient to cure the visible signs of the deficiency and to prevent recurrence of the syn-

drome, it appears that this level of methyl linoleate is the daily requirement of the mouse for this essential fatty acid.

SUMMARY

Essential fatty acid deficiency has been studied in mice of the C57 black, C3H, and dba strains. The most characteristic symptoms were: dermatitis of the skin and extremities, scaliness of the ears, alopecia, a neck lesion, and retardation of growth.

Pre-depletion of the weanlings, produced by placing the mother on a deficient diet during the lactation period, had a marked effect on the time of development of the symptoms.

The dba strain was found to be more sensitive to the deficiency than the C3H or C57 strains. Prophylactic and curative experiments indicate that under the experimental conditions used 5 mg of methyl linoleate per day satisfy the requirement of the mouse for linoleic acid.

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ASCORBIC ACID NUTRITURE IN THE HUMAN

I. TYROSINE METABOLISM AND BLOOD LEVELS OF ASCORBIC ACID DURING ASCORBIC ACID DEPLETION AND REPLETION¹

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ONE FIGURE

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Data as to dietary intakes and blood serum levels of ascorbic acid of a large population group have been compiled during recent nutritional survey studies in the Northeastern region.² Such data yield information concerning the ascorbic acid nutriture of the group at the time of the study, but without more detailed investigations they do not yield information concerning the past ascorbic acid nutriture of the group. The ascorbic acid content of the white cells and platelets is thought to be a more adequate measure of the tissue content of the vitamin. Investigations of this relationship have been made by Crandon et al. ('40), Butler and Cushman ('40), Lowry, Bessey, Brock and Lopez ('46), and the Medical Research Council of England ('48). Lowry and co-workers, in their study, which involved 100 subjects maintained for 8 months on diets containing varied amounts of ascorbic acid, showed the presence of a direct relationship between ascorbic acid concentration in serum and white cells at the end of the

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The authors wish to express their sincere appreciation to Katherine J. Newman for her aid in the preparation of this manuscript.

²Bulletins on the *dietary findings* and *chemical findings* of the Nutritional Status Project will be published by the Northeast Region NE-4 group.

study period. Such a relationship might enable one to assess the level of past ascorbic acid nutriture from the more easily obtained serum values, unless the individual had a highly erratic intake of the vitamin. It was thought, however, that more information was needed concerning the relationship between serum and white cell values determined at frequent intervals while subjects were ingesting a diet deficient in ascorbic acid.

In addition, it was considered important to investigate the urinary excretion of "tyrosyl" compounds, because this excretion might give early indication of ascorbic acid depletion. Various workers (Sealock and Silberstein, '39, '40; Levine, Marples and Gordon, '39) have reported urinary excretion of abnormal amounts of tyrosine metabolites by guinea pigs and premature infants deficient in ascorbic acid. Rogers and Gardner ('49) have made similar observations with respect to scorbutic adult humans. To our knowledge, no study involving tyrosine metabolism during an induced sub-optimum ascorbic acid intake in the adult human has been reported in detail.

This report is concerned with the urinary excretion of "tyrosyl" compounds and the ascorbic acid level of serum and white cells of 10 adult humans ingesting a diet containing 7 mg or less of vitamin C.

EXPERIMENTAL

Five men and 5 women volunteered to serve as subjects. They ranged between 21 and 32 years of age and on the basis of medical examination were judged to be in good physical condition.

The menus were planned on a three-day basis and were repeated throughout the experimental period of 82 days. A "core" diet consisting of eggs, meat (beef, pork, fowl), milk, stewed prunes, and canned pears was ingested by each subject. Cereal products and butter were added in various amounts to meet each subject's energy needs. Sugar, special hard candies, and soft drinks were allowed ad libitum but

in known amounts. With the exception of ascorbic acid, the diet was considered to be adequate in known dietary essentials for the human. The daily intake of ascorbic acid was 15 mg or less during the first two weeks; by treating the milk with heat, the ascorbic acid intake was reduced to 7 mg or less per day during the remainder of the depletion period. The diet contained variable amounts of pteroylglutamic acid, a compound which is known to inhibit hydroxyphenyluria when given in a high enough dose (Govan and Gordon, '49; Morris, Harper and Goldbloom, '50). It is doubtful that the amount present in an ordinary diet would have a retarding effect.

During the morning of the first day of the study, fasting samples of venous blood were withdrawn. After one week a balance period of 6 days was started. The subjects received 5 gm of L-tyrosine with each noon and evening meal during the balance period (a total of 10 gm per day). Complete urine and fecal collections were made, and at least one fasting sample of blood was withdrawn during each metabolic period.

Other balance studies, of at least 6 days each, were made after the subjects had been ingesting the diet for 31, 54 and 73 days. The last 4 days of the study (79 to 82 days) were devoted to saturation tests. During this time each subject received 800 mg of L-ascorbic acid (in 4 200-mg doses ingested at least 4 hours apart) in addition to the usual menu. The 10-gm dose of L-tyrosine was fed during this period also.

The amount of L-ascorbic acid in urine was determined by the method of Evelyn, Molloy and Rosen ('38). The total excretion of hydroxyphenyl compounds, designated as "tyrosyl" compounds, was measured by the method of Medes ('32), which was adapted to the Evelyn colorimeter. The reducing power of the urine was determined as hydroquinone equivalents according to the method of Briggs ('22) adapted to the Evelyn colorimeter. This method measures *p*-hydroxyphenylpyruvic acid, but it is not specific and will also measure homogentisic acid, ascorbic acid, and other reducing sub-

stances. The method of Peters ('42) was used to determine the creatinine content of the urine.

Determinations of ascorbic acid in serum and white cells were made according to the methods devised by Bessey and Lowry and described by Gyorgy ('50) and outlined in Northeast Regional Publication No. 5 ('51).

The method of Roe and Kuether ('43) was used to determine the total ascorbic acid content of the diet after the vitamin had been extracted from the foods by the method of Loeffler and Ponting ('42).

RESULTS

Blood

The average serum and white cell values of ascorbic acid for the 10 subjects during ascorbic acid depletion and repletion are given in table 1. After one week of the low ascorbic acid diet, the serum values had fallen approximately 50%, from 1.1 ± 0.048 to 0.6 ± 0.048 mg %. After 31 days of depletion, the average ascorbic acid level had fallen another 50%, to 0.3 ± 0.039 mg %, and it remained relatively con-

TABLE 1

Average levels of ascorbic acid in the blood and urine, and of "tyrosyl" compounds and hydroquinone equivalents in the urine, of 10 human subjects during ascorbic acid depletion and repletion

DAYS ON DIET	BLOOD ASCORBIC ACID		URINE		
	Serum	White cells	<i>l</i> -ascorbic acid	"tyrosyl" compounds	Hydroquinone equivalents
	mg %	mg %	mg/24 hours	mg/24 hours	mg/24 hours
Pre-diet	1.1 ± 0.048 ¹				
7 through 12	0.6 ± 0.048	17.1 ± 1.55	5.2 ± 1.0	420 ± 59	110 ± 42
31 through 36	0.3 ± 0.039	13.0 ± 0.92	5.2 ± 0.7	521 ± 91	160 ± 19
54 through 60	0.3 ± 0.047	10.9 ± 0.61	4.8 ± 0.9	482 ± 71	167 ± 17
73 through 78	0.2 ± 0.033	11.5 ± 0.32	4.4 ± 0.5	465 ± 61	201 ± 30
79 through 82 ²	2.3 ± 0.33	33.4 ± 1.91	165 ± 64	431 ± 61	³

¹ Mean \pm standard error.

² Repletion period; each subject received 800 mg of *l*-ascorbic acid per 24 hours.

³ Ascorbic acid interferes with this determination.

stant during the remainder of the depletion period. White cell ascorbic acid fell less rapidly. At the end of the first week the white cells contained 17.1 ± 1.55 mg % of ascorbic acid. At the end of one month of depletion there was a decrease of approximately 23% in the cell content. The total decrease of ascorbic acid in the white cells for the 78-day deficiency period was approximately 33%, from 17.1 to 11.5 mg %. The values of 0.2 ± 0.033 and 11.5 ± 0.32 mg % for serum and white cells, respectively, found at the end of 78 days, are almost identical with those of Lowry et al. ('46), who reported values of 0.18 ± 0.01 and 11.9 ± 0.4 mg % at the end of 8 months of depletion.

Of the 23 instances when serum values were less than 0.4 mg % only one white cell value was greater than 14 mg % (fig. 1). No value for white cells under 14 mg % was found when serum values were greater than 0.4 mg %. Both high and low white cell values occurred at a serum level of 0.4 mg %. These are lower white cell values relative to serum values than those reported by Lowry et al. ('46).

Urine

The urinary excretion of ascorbic acid remained low during ascorbic acid depletion (table 1). "Tyrosyl" compound excretion increased from an average of 420 ± 59 mg in the first metabolic period to 521 ± 91 mg per 24 hours during the second period. There was relatively little change in the total amounts of the compound excreted per period as the subjects became more depleted in ascorbic acid, but there was a variation in the daily excretion of "tyrosyl" compounds. The highest daily excretion of "tyrosyl" compounds by a subject was 1,406 mg; the following day the subject excreted less than 1,000 mg. The values found in this study would not be considered indicative of abnormal tyrosine metabolism on the basis of the work of Rogers and Gardner ('49).

There was a trend toward an increase in the reducing power of the urine as the subjects became more deficient in vitamin C. The reducing power of the urine, expressed as

hydroquinone equivalents, increased from an average of 110 ± 42 mg in the first balance period to 160 ± 19 mg per 24 hours in the second balance period (table 1). There was no important change in the total amount excreted in the third

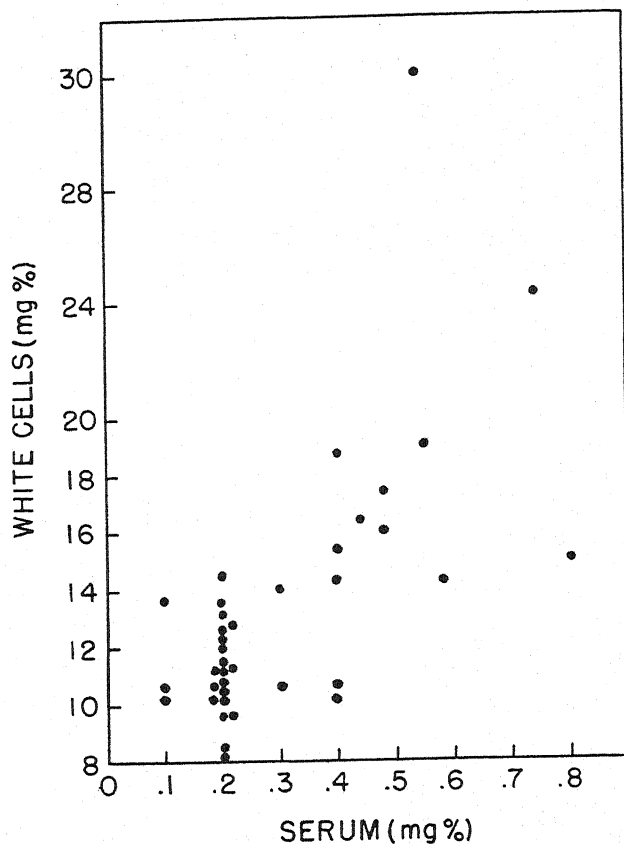


Fig. 1 Comparison of the ascorbic acid concentrations in serum and white blood cells of 10 subjects during depletion of ascorbic acid.

period. During the 4th period the average excretion of hydroquinone equivalents increased to 201 ± 30 mg per 24 hours. The daily excretion of these compounds showed a progressive increase during the 4th metabolic period until the third day. The excretion decreased slightly the next two days and in-

creased again on the last day. Because ascorbic acid contributes to the reducing power of the urine, it was not possible to ascertain whether the hydroquinone equivalents decreased during the subsequent realimentation period. The greatest excretion of reducing substances by any one subject, 706 mg during the 4th period, is above the values found for normal subjects by Rogers and Gardner ('49). The average values reported in table 1, however, are well within the normal range.

Ascorbic acid repletion

All subjects achieved normal values for serum and white cell vitamin C when they were given 800 mg of the vitamin daily for 4 days (table 1). All but two subjects, D.B. and M.G., showed evidence of tissue saturation at the end of the 4th day, as indicated by urinary excretion of approximately 50% of the repletion dose (table 2). The 8 subjects considered satu-

TABLE 2

A 4-day ascorbic acid repletion study on 10 human subjects maintained for 78 days on a low ascorbic acid intake (7 mg or less/day)¹

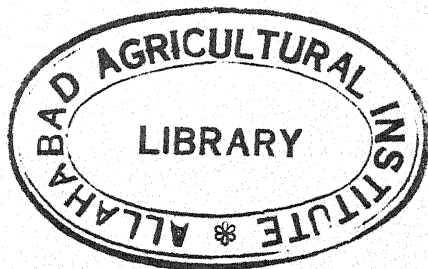
SUBJECTS	Day	URINARY ASCORBIC ACID				ASCORBIC ACID RETAINED	WHITE CELL ASCORBIC ACID	
		1	2	3	4		Pre-repl.	Post-repl.
		<i>mg/24 hours</i>				<i>mg</i>	<i>mg %</i>	<i>mg %</i>
D.B. ²	12.5	218	311	290		635 ³	13.2	30.5
R.B.C.	3.3	10	277	487		1,171	12.0	40.0
R.C.	4.0	7	170	482		1,265	11.4	31.0
B.E.	9.0	15	101	434		1,177	9.6	29.2
B.G.	4.0	46	417	474		955	10.2	26.5
M.G.	8.0	9	8	105		290 ³	10.8	35.4
R.H.	6.0	8	76	370		1,020	10.8	31.1
B.L. ⁴	5.0	6	38	394		1,133	10.8	29.2
R.L.	3.0	6	116	389		1,042	13.2	33.8
R.R.	4.0	116	529	626		1,231	12.6	47.1
		average				1,120		

¹ Unless noted otherwise, each subject was given 800 mg l-ascorbic acid/day for 4 days.

² Subject started on repletion one day earlier than others, given 600 mg l-ascorbic acid, excreted 4 mg.

³ Not included in average values.

⁴ Received 600 mg l-ascorbic acid on second day.



rated retained from 0.966 to 1.265 gm of ascorbic acid over the 4-day period. The retention each day was calculated by subtracting the ascorbic acid excreted that day from the amount excreted at a time when no retention was taking place; e.g., at saturation (Lowry et al., '46).

Subjective symptoms

At the end of 60 days of deficiency, two subjects reported bleeding gums and many of the subjects exhibited increased irritability and complained of undue fatigue. No subject reported having an abnormally high number of respiratory infections. After 76 days of depletion one subject, D.B., had severe skin outbreaks similar to hives and developed small petechiae when these eruptions were scratched. The skin outbreak disappeared after this subject received 600 mg of ascorbic acid on the 77th day and 800 mg daily on each of the succeeding 4 days. It is interesting to note that this same subject was not considered saturated at the end of the experiment, although she had received 3.8 gm of ascorbic acid orally by this time (table 2).

Although the above symptoms were encountered while the subjects were ingesting the diet low in ascorbic acid, the authors do not wish to imply that a cause and effect relationship exists. The symptoms are merely noted as part of the observations made.

DISCUSSION

The ingestion of a diet containing 7 mg or less of ascorbic acid per day by 10 human subjects for 78 days resulted in a lowering of the serum and white cell ascorbic acid content. After 31 days on the deficient diet, the serum ascorbic acid remained at essentially the same low level. White cell ascorbic acid reached its lowest value after the 54th day on the diet.

Similar to the results of Lowry et al. ('46), there seemed to be a relationship between ascorbic acid concentration in the serum and white cells. The present data indicate that with

a serum value *greater* than 0.4 mg %, the subject has a white cell ascorbic acid content of 14 mg % or more. When the serum value is less than 0.4 mg % the cell value is probably below 14 mg % and the adequacy of the ascorbic acid nutriture of the person might be questioned. Thus it appears that some evaluation of past ascorbic acid nutriture may be made on the basis of serum values alone, if the individual's intake of the vitamin has not varied drastically.

During the 78-day period of ascorbic acid deficiency, the only evidence that might be interpreted as being indicative of abnormal tyrosine metabolism was the increased excretion of reducing substances noticed during the 4th period. These values, however, are still within the normal ranges reported by Rogers and Gardner ('49). It is possible that our subjects never became depleted enough in ascorbic acid to manifest abnormal excretion values, although it is known that guinea pigs and infants respond early in ascorbic acid depletion to tyrosine administration.

The work of Levine, Marples and Gordon ('41) indicates that the dosage of tyrosine used is important in eliciting an abnormal response. Rogers and Gardner ('49) gave their scorbutic subjects 20 gm of tyrosine per day. Our subjects received 10 gm per day, or approximately 0.15 gm per kilogram of body weight. While this amount is low when compared with that used by other experimenters, it represents nearly two and one-half times the amount of protein-tyrosine found in a normal diet containing 100 gm of protein (Steele et al., '50). In view of the capability of intestinal organisms to decarboxylate tyrosine and form a toxic amine, it seemed inadvisable to give a higher dose.

SUMMARY

Ten adult humans were fed a diet containing 7 mg or less of vitamin C for 78 days, and the urinary excretion of "tyrosyl" and reducing compounds, and the ascorbic acid level of serum and white cells were determined.

Average serum and white cell ascorbic acid values fell to 0.3 and 13 mg %, respectively, at the end of 31 days of depletion. After 78 days they averaged 0.2 and 11.5 mg %, respectively. These levels returned to normal when the subjects were repleted with 800 mg of ascorbic acid daily for 4 days.

Of the 23 instances when serum values were less than 0.4 mg % only one white cell value was greater than 14 mg %. When serum values greater than 0.4 mg % were encountered (8 cases), no values under 14 mg % were found for white cells.

On the basis of the results of this study, low blood serum ascorbic acid values (less than 0.4 mg %) would indicate a past history of poor ascorbic acid nutriture and an inadequate or deficient intake of this vitamin.

There was an increase in the reducing power of the urine as the subjects became more depleted of ascorbic acid, but the urinary excretion of "tyrosyl" compounds and of reducing substances did not reach abnormal levels during the depletion period, even though 10 gm of free tyrosine were given orally to each subject during the 6 days of each metabolic period.

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PRODUCTION OF MULTIPLE CONGENITAL ABNORMALITIES IN YOUNG BY MATERNAL PTEROYLGLUTAMIC ACID DEFICIENCY DURING GESTATION ¹

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THIRTEEN FIGURES

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Previous studies have demonstrated that when succinyl-sulfathiazole (SST) is incorporated in a purified diet lacking pteroylglutamic acid (PGA), the vitamin is essential for reproduction in the rat (Nelson and Evans, '47, '49). Such a vitamin deficiency was not pronounced in its effects, as shown by the necessary duration of deficiency required to disturb reproduction, and even under these conditions there were only 26% resorptions. However, the addition of 0.5% of a "crude" PGA antagonist (α -methyl-PGA) produced an immediate effect, so that resorption invariably occurred when rats were placed on such a diet the day of breeding (Nelson and Evans, '49). Macroscopic studies of implantation sites and fetuses indicated that under such conditions the death of the fetus occurred on or before the 11th day of gestation (unpublished data). The present communication reports:

¹Presented in part before the 63rd meeting of the American Association of Anatomists, April 1950 (Asling and Nelson, '50; Nelson, Asling and Evans '50) and at the autumn meeting of the National Academy of Sciences, November 1951 (Evans, Nelson and Asling, '51). This research was aided by grants from the Board of Research and the Department of Agriculture of the University of California and the Roche Anniversary Foundation.

(1) the occurrence of resorptions when the deficiency is instituted as late as 9 days after breeding; and (2) the production of multiple congenital abnormalities in the young when the deficiency is instituted 10 to 13 days after breeding.

EXPERIMENTAL

Normal female rats (Long-Evans strain), three to 4 months of age, were bred with normal males and placed on the purified PGA-deficient diet containing SST and x-methyl-PGA at various times during gestation. Groups of animals were started 7, 9, 10, 11, 12, 13, and 15 days after breeding.² Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation has occurred; all rats were weighed at regular intervals. The animals were kept on screens until the day before littering was expected to occur. All rats were autopsied a few hours after parturition or the day before expected parturition (day 21). All fetuses were carefully examined for macroscopic abnormalities and the uterus was checked for the presence of normal or resorbing implantation sites.

The PGA-deficient diet containing the antagonist was the same as that used previously (Nelson and Evans, '49). It consisted of 24% alcohol-extracted casein, 62.5% sucrose, 8% hydrogenated cottonseed oil,³ 4% salts,⁴ 1% SST, and 0.5% x-methyl-PGA. Crystalline vitamins per kilogram diet were: 300 μ g *d*-biotin, 5 mg methyl-1,4-naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg *p*-aminobenzoic acid, 20 mg nicotinic acid, 50 mg *d*-calcium

² Many authors differ in timing the days of gestation. In this laboratory the day of finding sperm is called the day of breeding and is considered to be day zero. Wilson and Karr ('51) have recently pointed out that the morning of finding sperm probably represents a fair approximation of the mean time of fertilization and they have, therefore, used this system of timing in their studies on the effects of irradiation. All previous papers by Warkany and by Wilson on congenital abnormalities used a different system of timing, by designating the day of finding sperm as day one.

³ Crisco or Primex.

⁴ Salts 4 of Hegsted et al. ('41).

pantothenate, 400 mg inositol, and 1.0 gm choline chloride. Additional groups of animals received high levels of ascorbic acid and vitamin B₁₂, separately or combined. All rats received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units of vitamin D, 6 mg synthetic alpha-tocopherol, and 650 mg corn oil.⁵ The "crude" PGA-antagonist (α -methyl-PGA) used was that described by Franklin et al. ('47).

Control animals maintained on the identical diet supplemented with a high level of the synthetic vitamin, 50.5 mg synthetic PGA per kilogram diet, showed no impairment of reproductive performance and no macroscopic or microscopic abnormalities in the young.

RESULTS

Table 1 summarizes the reproductive performances of rats placed on the PGA-deficient diet 7 to 15 days after breeding and maintained thus throughout the remainder of gestation. The data for rats placed on the deficient diet the day of breeding are included for comparison. The groups averaged 102 to 119 days of age and 212 to 226 gm in body weight on the day of breeding. All groups showed implantation, with a normal number of implantation sites per rat (9 to 11) and the normal appearance of vaginal erythrocytes on the 13th day of gestation. The maternal weight gain during gestation increased from 21 gm when the deficiency was instituted on the day of breeding up to the normal maximum of 100 gm or more when the deficiency was instituted 12 days or more after breeding. All bred rats were normal in appearance and in good condition throughout gestation. In particular, no severe anemia, leukopenia, or granulocytopenia was noted.

Under the experimental conditions, instituting the deficiency 7 or even as late as 9 days after breeding invariably results in 100% resorptions (table 1). In marked contrast are the results obtained when the deficiency is instituted only one day later, day 10. Forty per cent of the animals litter, although

⁵ Mazola.

TABLE 1
Occurrence of resorption or abnormal young when mothers are submitted to PGA-deficiency during gestation

PERIOD OF DEFICIENCY (days of gestation)	NO. OF RATS BREED	WT. CHANGE DURING GESTATION	RESORPTIONS	LITTERS	IMPLANTATION SITES PER RAT			YOUNG	
					Total ¹	No. normal	Wt.	Dead	Abnormal
		gm	%	%			gm	%	%
1-21	47	+ 21	100	0	10.5	0		None	
7-21	20	+ 46	100	0	9.9	0		None	
9-21	22	+ 61	100	0	9.6	0		None	
10-21	22	+ 70	59	41	11.0	3.0	3.8	100	100
11-21	47	+ 90	4	96	10.2	7.8	4.0	100	95
12-21	23	+ 110	0	100	10.0	9.2	4.5	97	65
13-21	34	+ 100	0	100	10.1	9.5	4.3	56	30
15-21	20	+ 106	0	100	10.3	9.6	5.3	6	0
					PGA controls ²				
1-21	21	+ 116	0	100	11.3	10.6	5.6	4	0
10-21	21	+ 112	0	100	10.0	8.9	5.7	2	0

¹ The total number of implantation sites per rat equals the number of normal sites with living young plus the number of resorbing sites; all sites were checked by macroscopic examination at autopsy.

² Animals fed the identical diet supplemented with 50.5 mg synthetic pteroylglutamic acid per kilogram.

the number of young per litter and their weight at birth are markedly decreased. Delaying the deficiency until day 11 results in practically normal littering (96% litters), although the weight of the young at birth and sometimes the number of young per litter are decreased. Instituting the deficiency still later in gestation, i.e., day 12, 13, and 15, results in 100% litters with no decrease in the number of young per litter, although the birth weight of the young was less than 5 gm except for the group started on day 15.

TABLE 2

Incidence of macroscopic abnormalities encountered in young from mothers submitted to PGA-deficiency during gestation

PERIOD OF DEFICIENCY (days of gestation)	NO. OF YOUNG	ABNORMAL YOUNG	EDEMA	CLEFT PALATE	SYNDACTYLISM		OTHER ABNORMALITIES
					Fore-paws	Hind-paws	
10-21	39	100	100	100	65	70	Kidneys, lungs
11-21	126	95	95	92	25	50	Kidneys, lungs
12-21	109	65	90	2	0	0	Kidneys
13-21	70	30	slight	0	0	0	Kidneys
15-21	96	0					

Description of abnormal young

The young produced by the mothers given the deficient diet on days 10 to 21 or 11 to 21 are very unusual in appearance and exhibit multiple congenital abnormalities (figs. 1 and 2, tables 1 and 2). In the first group of animals studied, all young were either dead when examined or missing. This prompted examination of the next series of mothers on the day before parturition, day 21. These 21-day-old fetuses were apparently living, as judged by a beating heart and reflex movements, but the lungs did not expand. Ninety-five to 100% of these fetuses were markedly abnormal macroscopically and were characterized by marked edema, anemia, and multiple skeletal and visceral abnormalities. The edema (figs. 1, 2, 5 through 8), which was primarily subcutaneous, resulted in swollen, misshapen, translucent bodies, in some

of which were whitish areas dorsally. The anemia was obvious macroscopically. Hematological studies revealed hemoglobin values as low as 1 to 3 gm per 100 ml and RBC counts of 250,000 to 500,000, in comparison with control values of 10 to 13 gm hemoglobin and two to three million RBC. Other abnormalities obvious on external inspection included cleft palate, marked retardation in facial development, brachydactylism or syndactylism, and short or curled tails. When the animals were cleared and stained with alizarin red (figs. 3 and

TABLE 3

Effect of maternal PGA deficiency, days 11 to 21, on body and organ weights of 21-day-old fetuses¹

	CONTROLS	PGA-DEFICIENT	% of controls
	<i>mg</i>	<i>mg</i>	
Body weight	5,500	3,200	58
Heart	25	18	70
Adrenals	2.7	1.7	63
Testes	4.6	2.7	59
Eyeballs	24	14	57
Brain	215	105	49
Liver	395	186	47
Stomach	38	11	30
Spleen	11	2.3	21
Intestines	152	30	19
Thymus	8	1.5	19
Lungs	108	18	16
Kidneys	49	5	10

¹ These averages are based on 12 control and 14 deficient fetuses.

4), skeletal abnormalities associated with these were revealed and, in addition, retardation in skeletal maturation, deformed or misshapen bones, and the absence of certain ossification centers. Virtually no part of the skeleton escaped damage. In addition, some rats showed alizarin-red staining of the kidneys, the aorta, and the dorsal whitish areas. These skeletal changes are reported in detail elsewhere (Asling, Nelson and Evans, '52).

At autopsy all organs of these fetuses were decreased in weight in comparison with those of the controls. Table 3

reveals that the organs most markedly affected were: the kidneys (10% of normal weight), the lungs (16%), and the thymus, intestines, spleen and stomach (20 to 30% of normal weight). All other organs were 50 to 70% of normal weight and in proportion to the decreased body weight, which was 58% of normal. Macroscopically, the kidneys were extremely small in size, pale, and practically white in color, with occasional cystic pelvises. The lungs were also extremely small in size and non-expanded.

Histological studies (to be reported later) revealed a marked retardation in development of kidneys, lungs and skin and a less marked delay in development of practically all other organs and tissues. The lungs were underdeveloped and non-expanded but showed irregular cystic dilatation of many bronchioles (figs. 5 through 8). Both epidermis and dermis were thinner and less differentiated, while the tela subcutanea was tremendously expanded (figs. 5 through 8) and in it were widely scattered primitive connective tissue cells. The distension of this area was responsible for the swollen and misshapen appearance of the fetus (figs. 2 and 6). In the kidneys (figs. 9 through 11) the renal corpuscles and convoluted tubules were markedly underdeveloped, whereas the collecting ducts usually showed greater development and might even appear almost normal. Few connections between the secretory and excretory duct systems could be observed. Occasionally renal corpuscles, but more often the pelvis of the kidney, were markedly dilated and cystic. The eyes, likewise, exhibited retarded development and, more important, a high incidence of Morgagnian-type cataract (degeneration with vacuolization of the lens fibers; figs. 12 and 13).

Timing of the deficiency

The incidence and type of abnormalities varied with the time of instituting the deficiency (tables 1 and 2). Practically all young from mothers placed on the deficient diet on days 10

to 21⁶ or 11 to 21 showed some or all of these abnormalities. Marked edema, cleft palate and visceral abnormalities were present in 90 to 100% of the abnormal young. Although all abnormal young showed some skeletal defects other than cleft palate, syndactylism was higher in days 10 to 21 than in the 11 to 21 group. The hind paws were affected more frequently than the forepaws in the latter group. In contrast, only 65% of the young from mothers placed on the deficient diet one day later, day 12, showed abnormalities, and these were usually less marked. A variable amount of edema and some decrease in kidney size and development were present in 90% of the abnormal young of this group. Only 2% of these young exhibited cleft palates and no syndactylism was observed. Delaying the deficiency one more day, to day 13, resulted in only 30% macroscopically abnormal young; a slight edema was the principal abnormality, although the kidneys did not always attain normality in size. When the deficiency was instituted during days 15 to 21, no abnormal young were observed.

Additional experiments have demonstrated that the timing of the deficiency is the primary factor in abnormal fetal development and mortality, rather than the total amount of PGA-antagonist ingested. The amount of antagonist ingested can be calculated from the level of antagonist in the diet and the duration of the deficiency, assuming an average daily food intake of 16 gm. (This deficiency has only slight effects on food consumption even when instituted on the day of breeding; Nelson and Evans, '49.) The group deficient days 9 to 21, receiving the usual antagonist level (0.5%), consumed approximately 960 mg of PGA-antagonist and showed 100% resorptions. In contrast, a group deficient days 11 to 21, receiving a higher antagonist level (0.6%) which resulted in ingestion of approximately the same amount, 960 mg PGA-antagonist, showed only 17% resorptions. Another group deficient days 10 to 21, receiving a lower level (0.4%), con-

⁶ Additional abnormalities of the urogenital and cardiovascular systems were observed in the group made deficient on days 10 to 21; these will be reported later.

sumed an estimated 700 mg antagonist, which resulted in 38% litters, with 100% of the young exhibiting marked abnormalities; while the group deficient days 12 to 21, receiving the usual level (0.5%), consumed an estimated 720 mg antagonist, which resulted in 100% litters with only 65% of the young showing abnormalities, and these were less severe.

A similar gradation was shown in the percentage of dead young from these groups, one-third to one-half of which were allowed to litter. Instituting the deficiency on day 10 or 11 resulted in 100% and on day 12 in 97% dead young. In contrast, delaying the deficiency to day 13 resulted in a marked decrease to 56% dead young, and a further delay to day 15 in only 6% dead young, a close approximation to the maximum of 2% dead young for stock animals or 2 to 4% dead young for controls receiving high levels of the synthetic vitamin.

DISCUSSION

The data presented show the severity of pteroylglutamic acid deficiency as a teratogenic agent for the rat. Multiple congenital abnormalities in extremely high incidence, 95%, characterized the young from mothers placed on the deficiency regimen 11 days after breeding. The observed abnormalities, i.e., anemia, edema, skeletal malformations such as cleft palate and syndactylism, retarded visceral development which was especially noticeable in the kidneys and lungs, and Morgagnian-type cataracts, are in some respects similar to or identical with congenital abnormalities reported for other vitamin deficiencies in the rat, but in other respects they are unlike any malformations previously reported.

The skeletal malformations we have observed in this study are similar to but more extensive than those reported for riboflavin deficiency in the rat (Warkany and Nelson, '41). The timing for production of these skeletal anomalies is, however, similar for both deficiencies, i.e., we have found the critical time for starting the PGA-deficiency to be between the 11th and 12th days whereas Warkany et al. ('42) found that the critical time for vitamin (B₂) supplementation of the

diet to be between the 12th and 13th days.⁷ It is a reasonable assumption that the institution of the PGA-deficient diet will require at least one day to affect the fetus. The edema observed is similar but apparently much more extensive than that reported for deficiencies of either vitamin A (Wilson and Warkany, '48) or pantothenic acid (Boisselot, '49, '51). The retardation of lung development is similar to that reported for vitamin A deficiency (Wilson and Warkany, '48), although no cystic enlargement was observed in vitamin A deficiency. The retardation of kidney development is macroscopically similar to hypoplasia of the kidney encountered in the same studies of vitamin A deficiency but differs in affecting primarily the renal corpuscles and convoluted tubules (the secretory portion) instead of the collecting tubules or excretory portion of the kidney. Morgagnian-type cataracts of the eyes have not been reported previously for any vitamin deficiency, adult or fetal, in the rat though other congenital abnormalities of the eyes are associated with vitamin A deficiency (Warkany and Schraffenberger, '46) and pantothenic acid deficiency (Boisselot, '49, '51). The marked anemia observed is similar to that produced by PGA deficiency in the adult rat and may, therefore, be classified as a sign of fetal PGA-deficiency, just as keratinization of the fetal urogenital tract is a sign of fetal vitamin A deficiency (Wilson and Warkany, '47) and fetal cataract of fetal tryptophan deficiency (Pike, '51). Giroud and Boisselot ('51) have mentioned the occurrence of anemia in young from PGA-deficient mothers.

Previous reports have been made on congenital abnormalities, other than those reported in this study, resulting from maternal PGA-deficiency in the rat and other animals. Richardson and Hogan ('46) reported a low incidence (2%) of hydrocephalus in young from rats maintained for long periods on purified diets. Further studies have raised the incidence to more than 20% and have indicated that both vitamin B₁₂ and PGA are concerned (O'Dell et al., '51). A similar pos-

⁷ Warkany's timing is corrected in accordance with identifying the day of finding sperm as day zero, corresponding with the timing used in this paper.

sible double deficiency is that reported by Ross et al. ('44) for swine maintained on vegetable protein diets; skeletal anomalies such as syndactylism, talipes and dyostosis were noted together with edema, eye abnormalities or blindness, and paralysis agitans. By injections of various PGA-antagonists, Karnofsky et al. ('49) produced vascular abnormalities, absence of feathers, limb and beak abnormalities, and exteriorization of the viscera in chick embryos. Likewise Sunde et al. ('50) have found that maternal PGA deficiency in hens resulted in perosis, deformed mandibles, parrot beak, or fetal death for chicks. Recently Giroud and Boisselot ('51) have briefly reported the occurrence of multiple congenital abnormalities in young from rats maintained on a PGA-deficient diet containing SST. Anemia, hemorrhage, retinal colobomas, harelip with cleft palate, oblique facial fissures, atrophy of the nasal fossae, ectocardia and gastroschisis were observed. These abnormalities are similar to those we have observed with a transitory maternal PGA-deficiency early in gestation (data to be published).

The similarity of many of the congenital abnormalities observed in this study of maternal PGA-deficiency to those reported for other teratogenic vitamin deficiencies in the rat raises the question of the specificity of the vitamin deficiency produced but, even more important, the question of the specificity of the teratogenic action of any vitamin deficiency. The importance of timing the deficiency in regard to fetal death, abnormal young, or normal young, and especially the fact that the timing for the skeletal abnormalities observed in this study was similar to that observed for similar anomalies produced by riboflavin deficiency, would seem to indicate that the timing of the deficiency is of more importance than the specific nature of the deficiency. Stockard ('21) has emphasized the importance of timing in experimental teratology. In regard to this study of PGA-deficiency, the action of the PGA-antagonist (x-methyl-PGA) can be reversed only by high levels of PGA; in addition, high levels of ascorbic acid or vitamin B₁₂ or both had no effect.

SUMMARY

Under the experimental conditions employed, instituting a deficiency of pteroylglutamic acid in the rat as late as 9 days after breeding invariably resulted in fetal death (resorption), whereas delaying the deficiency two days to 11 days after breeding resulted in 95% of the animals littering young with multiple congenital abnormalities. These young were characterized by a marked edema and anemia, multiple skeletal abnormalities such as cleft palate and syndactylism, retarded development of the viscera, especially kidneys and lungs, and Morgagnian-type cataracts of the eyes.

Instituting the deficiency between day 9 and day 11, i.e., 10 days after breeding, resulted in approximately 40% of the animals producing litters, consisting of a few markedly abnormal young. In contrast, delaying the deficiency to 12 or 13 days after breeding resulted in 100% litters, with the young having milder degrees of edema and visceral retardation and practically no skeletal abnormalities. Normal young resulted when the deficiency was not started until 15 days after breeding.

The similarity of some of the abnormalities observed to those previously reported for other vitamin deficiencies in the rat is discussed.

ACKNOWLEDGMENTS

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PLATE 1

EXPLANATION OF FIGURES

Comparison of external form and skeletal structure of 21-day-old fetuses from mothers maintained on the PGA-control or deficient diet from the 11th to the 21st day of gestation.

- 1 Control fetus ($\frac{1}{2}$ natural size).
- 2 Deficient fetus ($\frac{1}{2}$ natural size). Note edema, translucence and whitish plaques on dorsum.
- 3 Control fetus, cleared and stained with alizarin red S. $\times 3.2$.
- 4 Deficient fetus. $\times 3.2$. Note general stunting, disproportionately short mandible, reduced number of ossification centers (as in paws), deformed bones (scapula, ribs, radius, ulna, and tibia) and defective vertebral formation.

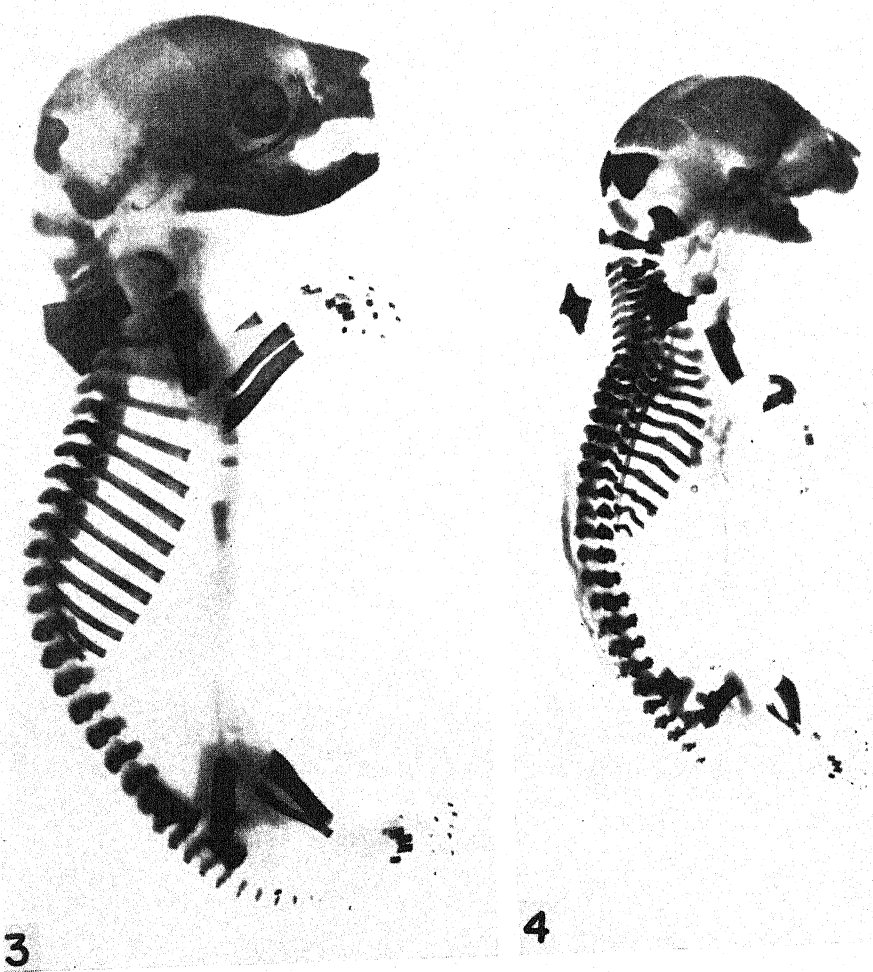
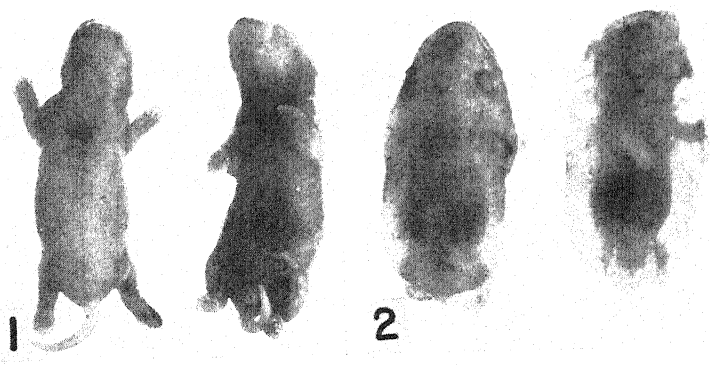


PLATE 2

EXPLANATION OF FIGURES

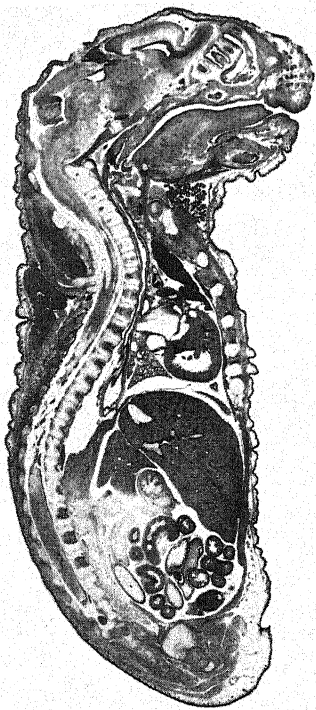
Histologic sections of 21-day-old fetuses from mothers maintained on the PGA-control or deficient diet from the 11th to the 21st day of gestation. Hematoxylin and phloxine B stain, 10 μ thickness.

5 Sagittal section of control fetus. $\times 2.4$.

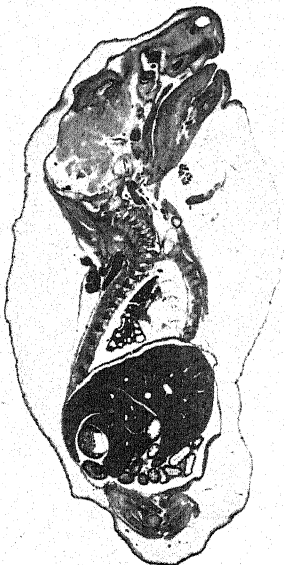
6 Sagittal section of deficient fetus. $\times 2.4$. Note thinner skin, edema of subcutaneous tissue and reduced size of all viscera. The lung is non-expanded but shows irregular cystic dilatations.

7 Transverse section of control fetus through upper part of thorax. $\times 4.2$. Arrow indicates normally expanded lung.

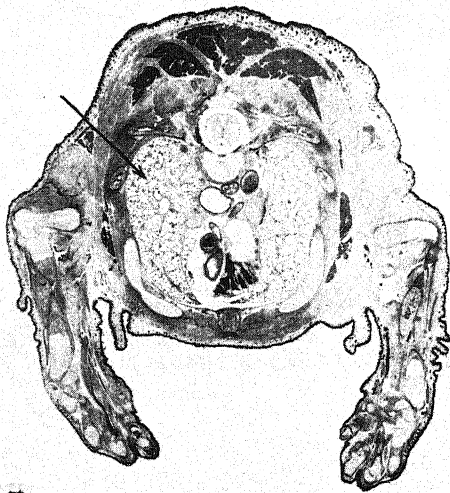
8 Transverse section of deficient fetus through same area. $\times 4.2$. Arrow indicates non-expanded lung tissue, still in fetal position. Note also the edema which extends into the extremities.



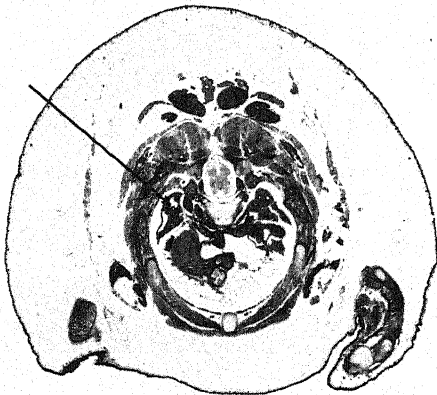
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PLATE 3

EXPLANATION OF FIGURES

Histologic sections through kidneys and eyes of 21-day-old fetuses from mothers maintained on the PGA-control or deficient diet from the 11th to the 21st day of gestation. Hematoxylin and phloxine B stain, 8μ (kidneys) to 10μ (eyes) thickness.

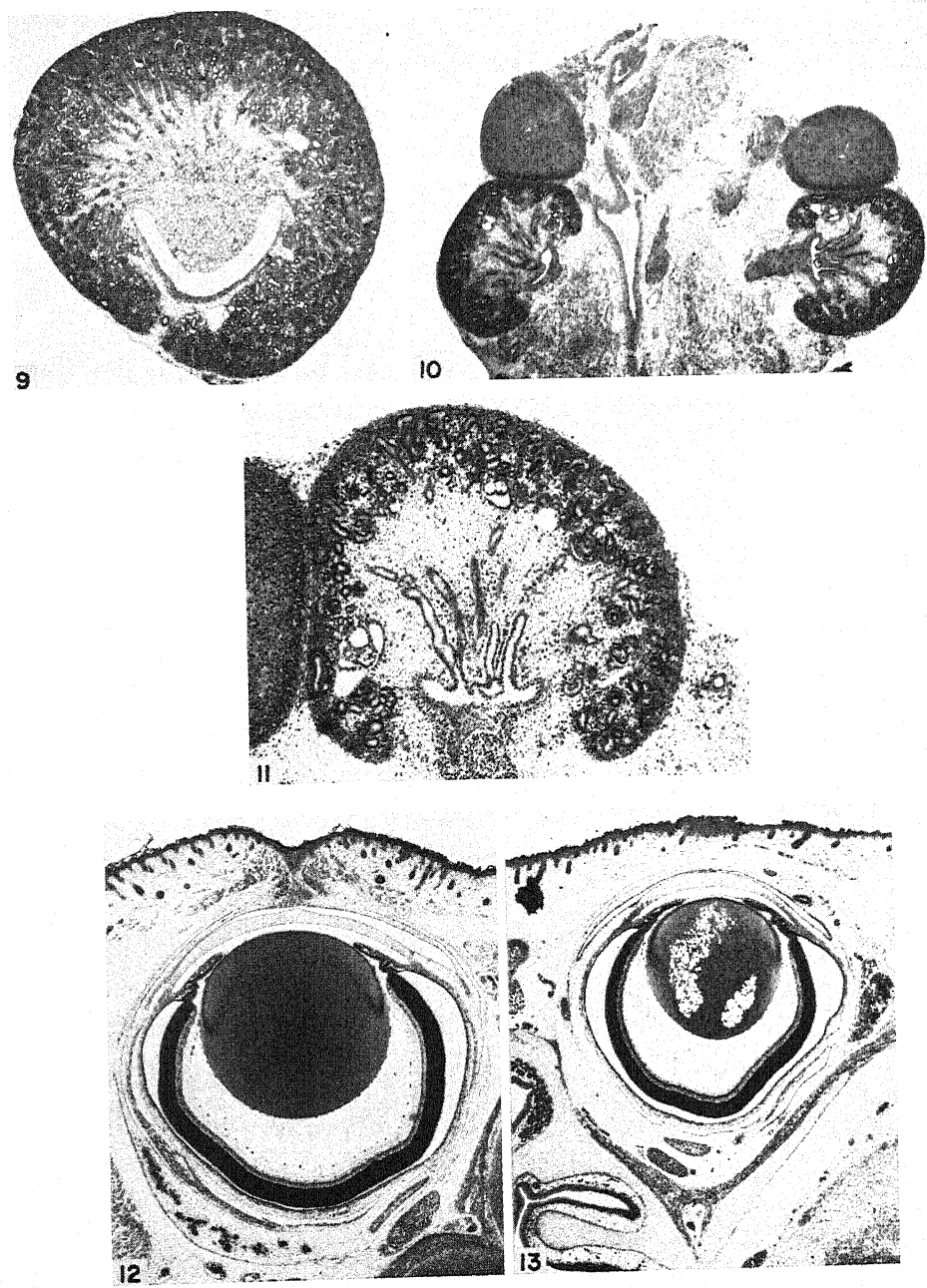
9 Kidney from control fetus. $\times 21$. Renal corpuscles are well-developed and majority have established connections with excretory ducts; kidney is ready to function.

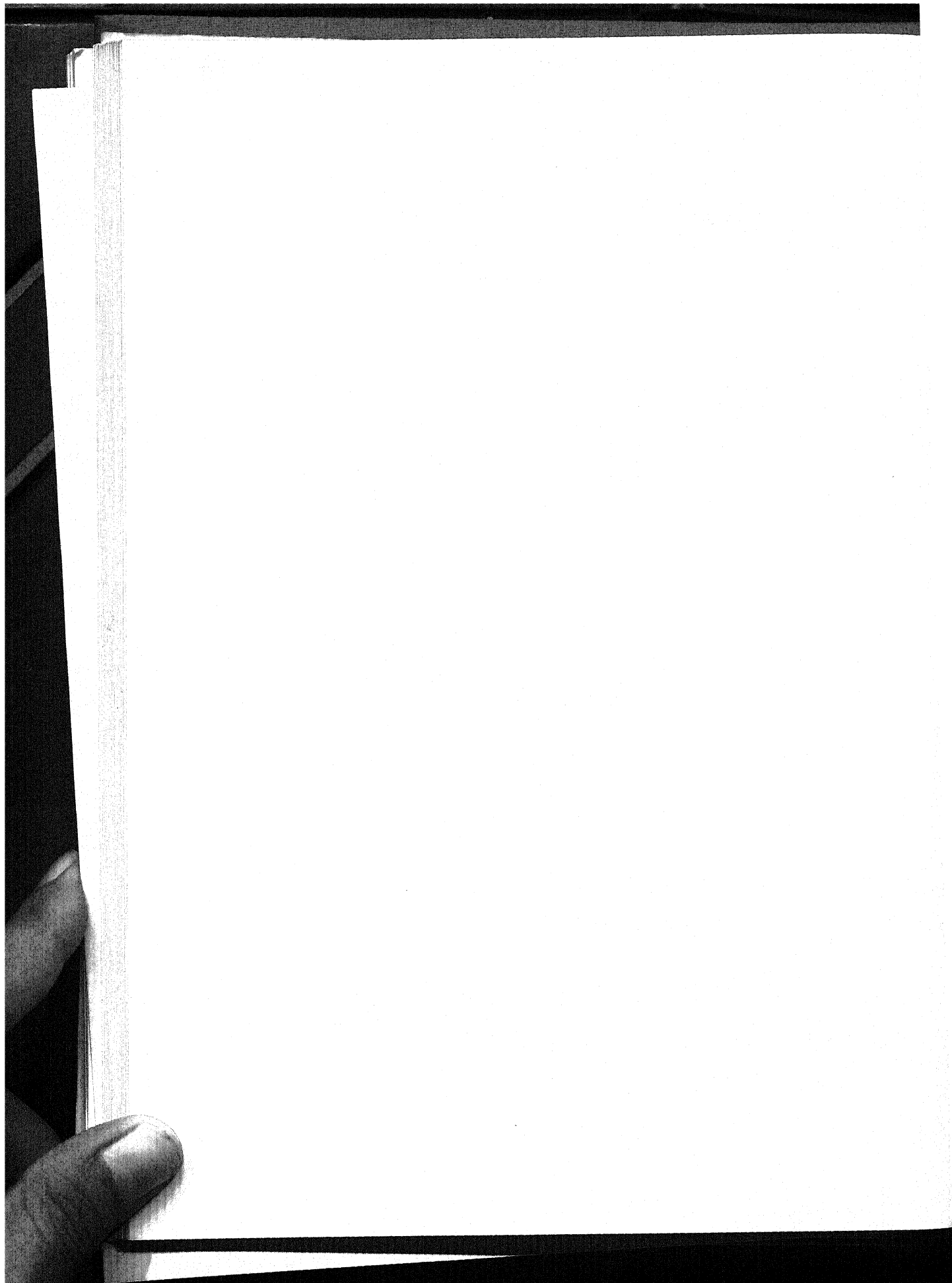
10 Kidneys and adrenals from deficient fetus. $\times 21$. Note marked reduction in kidney size, scarcely exceeding that of the adrenals, and retardation in development.

11 Higher magnification ($\times 55$) of right kidney seen in figure 10. This shows the marked reduction of glomerular development and lack of connections of renal corpuscles with excretory ducts. Only a few collecting ducts are seen in the medulla. Note cystic dilatations in convoluted tubules.

12 Eye of control fetus. $\times 21$.

13 Eye of deficient fetus. $\times 21$. Note reduction in size of eye and degeneration, with irregular vacuolation of the lens fibers. These changes in the lens are similar to those in congenital Morgagnian cataract observed clinically.





EFFECT OF THE LEVEL OF FAT IN THE DIET ON THE GROWTH PERFORMANCE OF DOGS¹

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THREE FIGURES

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Considerable interest has developed in the use of inedible fats in dry dog foods, particularly when such fats are in excess supply. McCay ('49) has stated that fats are well utilized by dogs; however, to our knowledge, a systematic study of the performance of dogs fed graded levels of fat in addition to a basal diet comprised of crude ingredients commonly used in commercial meals has not been reported. Although 10 to 15% fat is commonly added to purified diets for dogs, only 5 to 7% fat is used in commercial dry meals. In view of these considerations, it was of importance to evaluate the effect of adding fat, stabilized with an anti-oxidant, to practical diets on the growth rate, and food and caloric efficiencies of young dogs.

EXPERIMENTAL

Experiment 1

An experimental (basal) ration was formulated with ingredients commonly used in commercial dry dog meals to

¹ Journal Paper 53, American Meat Institute Foundation.

A report of work done under contract with the U. S. Department of Agriculture and authorized by the Research and Marketing Act. The contract is being supervised by the Eastern Regional Research Laboratory of the Bureau of Agricultural and Industrial Chemistry.

provide adequate amounts of the known growth factors for the dog as outlined by Michaud and Elvehjem ('44). As a further test of the formulated ration, a commercial dry meal was purchased directly from the manufacturer and compared with the experimental ration. The composition of the experimental ration and the proximate analysis of pooled samples of both rations are given in tables 1 and 2.

TABLE 1

COMPOSITION OF EXPERIMENTAL RATION ¹	AMOUNT
	%
Corn flakes	26.75
Wheat flakes	26.70
Soybean grits (HI-PRO-CON)	19.00
Meat and bone scrap	15.00
Fish meal (Menhaden)	3.00
Wheat germ meal (defatted)	5.00
Dried skim milk	2.50
A and D oil (Nopco xx-2250 U.S.P. units A, 400 A.O.A.C. units D/gm)	0.50
Iodized salt	0.25
Brewers' yeast (non debittered)	0.50
Riboflavin supplement (BY-500)	0.80
	100.00

¹ We are indebted to the Schlitz Brewing Co., Milwaukee, Wisconsin, for supplying the yeast, and to the A. E. Staley Manufacturing Co. for supplying some of the soybean grits used in this study.

TABLE 2

Proximate analysis of rations ¹

ANALYSIS	COMMERCIAL RATION	EXPERIMENTAL RATION
	%	%
Moisture	6.1	6.3
Protein (N \times 6.25)	25.7	29.1
Fat (ether extract)	5.9	3.7
Ash	6.9	6.7
Fibre	4.9	2.4

¹ Made by the Service Laboratory, American Meat Institute Foundation.

The added fat (choice white grease — a high grade of inedible pork fat) was stabilized by the addition of butylated-hydroxyanisole 0.02%, citric acid 0.01%, and propyl galate 0.005% (Kraybill et al., '49; Dugan et al., '50) to the melted fat at 80°C.

The rations were made up 60 days prior to feeding to approximate the shelf life of commercial meals and to investigate the development of rancidity during storage. Separate studies are being conducted on the effect of different anti-oxidant combinations on the stability of greases added to feeds (Neumer and Dugan, '52).

Registered cocker spaniel pups of mixed sex (average age of 12 weeks) were purchased, treated for intestinal parasites, dipped into a DDT solution for removal of external parasites, injected subcutaneously with anti-canine distemper serum, and individually housed in sterilized metal cages equipped with raised, expanded metal bottoms in a room thermostatically heated to 76°F. The dogs were fed the experimental ration during an initial two-week orientation period during which an infection (controlled through oral administration of antibiotics) occurred among some of the dogs.

At the termination of an additional two-week orientation period, the dogs were arranged into 6 feeding groups as evenly as possible (judged by litter, weight, sex and body conformation) and fed either the experimental ration (three dogs), experimental ration + 6% stabilized fat (three dogs), experimental ration + 6% stabilized fat with the caloric intake per dog restricted to the average caloric intake per dog for the group fed the experimental ration (4 dogs), experimental ration + 13.5% sucrose (sucrose equal in crude calories to 6% fat) (three dogs), commercial dog meal (three dogs), or commercial dog meal + 6% stabilized fat (4 dogs). The fat and sucrose were added at the expense of the entire ration. All dogs except those calorically restricted were fed ad libitum. All rations were fed dry and all groups were given water ad libitum. The group fed sucrose was included to determine if the effect of feeding fat could be attributed to

factors other than calories and to evaluate further any effect of diluting other nutrients (minerals, vitamins, proteins) associated with the addition of fat or sucrose to the rations.

The dogs were fed the rations for a 10-week period and weighed at weekly intervals. Food consumption records were obtained for each dog during the first through 8th week on experiment. The food and caloric efficiencies were calculated from these data.

Experiment 2

A second experiment was performed using female cocker spaniel pups (12 weeks of age) as the test animals. Female dogs were used in order to obtain reproduction and lactation data when they were fed various levels of fat. The results of the reproduction and lactation studies will be available at a later date. The basal ration, the stabilized fat added to the rations, and the treatment and housing of the dogs were identical with those of the first experiment. All rations were freshly mixed before feeding. Four groups of weanling female cocker spaniel pups were used (7 dogs in each group) and fed either the experimental ration, the experimental ration + 4% stabilized fat, the experimental ration + 8% stabilized fat or the experimental ration + 18% sucrose (sucrose equal in crude calories to 8% fat). The dogs were weighed at weekly intervals over a 10-week period. One dog in the group fed 8% added fat and two dogs fed the sucrose diet died during the first two weeks of the experiment from unknown causes. Food consumption records were obtained from the 4th through 7th week of the experiment. All dogs were fed and given water ad libitum and all rations were fed dry.

RESULTS AND DISCUSSION

The dogs fed the basal diet in experiment 1 averaged 371 gm gained per week, while the dogs fed the commercial ration averaged 334 gm gained per week (table 3). No differences in average food and caloric efficiencies were noted between these groups, indicating that the experimental ration used was

equal to, or better than, the commercial ration. The rates of gain and the food and caloric efficiencies of the groups fed 6% fat in addition to either the basal ration or commercial ration were equal to or slightly better than those for

TABLE 3

Effect of feeding different levels of fat on the rate of gain and the food and caloric efficiency of dogs

RATION	NO. OF DOGS	AVE. INITIAL WT.	AVE. GAIN/ WK.	FOOD EFFICIENCY ¹	CALORIC EFFICIENCY ²
<i>gm</i>					
Experiment 1					
Commercial ration	3	2,920	334	0.22	6.2
Commercial ration + 6% fat	4	2,490	383	0.24	6.2
Experimental ration	3	3,140	371	0.22	6.1
Experimental ration + 6% fat	3	3,410	443	0.26	6.8
Experimental ration + 6% fat ³	4	3,150	435	0.24	6.4
Experimental ration + 13.5% sucrose	3	2,990	402	0.22	6.2
Experiment 2					
Experimental ration	7	3,300	339	0.18 ± 0.011 ⁴	5.2 ± 0.32 ⁴
Experimental ration + 4% fat	7	3,180	360	0.17 ± 0.022	4.5 ± 0.55
Experimental ration + 8% fat	6	3,870	334	0.17 ± 0.026	4.4 ± 0.59
Experimental ration + 18% sucrose	5	3,310	328	0.15 ± 0.011	4.1 ± 0.30

¹ Grams gained per gram food consumed.

² Grams gained per 100 crude calories consumed.

³ Caloric intake restricted to that of the group fed the experimental ration (see text).

⁴ Standard error.

the groups fed the rations without added fat (figs. 1 and 2; table 3). The performance of the group fed sucrose was similar to that of the group fed the basal diet. The group fed the experimental ration + 6% fat with the caloric intake restricted to the caloric intake of the group fed the experi-

mental ration did not consume the calories allotted and, therefore, can be considered as having been fed *ad libitum*. Excellent agreement was found for the average rates of gain, food efficiencies, and caloric efficiencies between the two groups

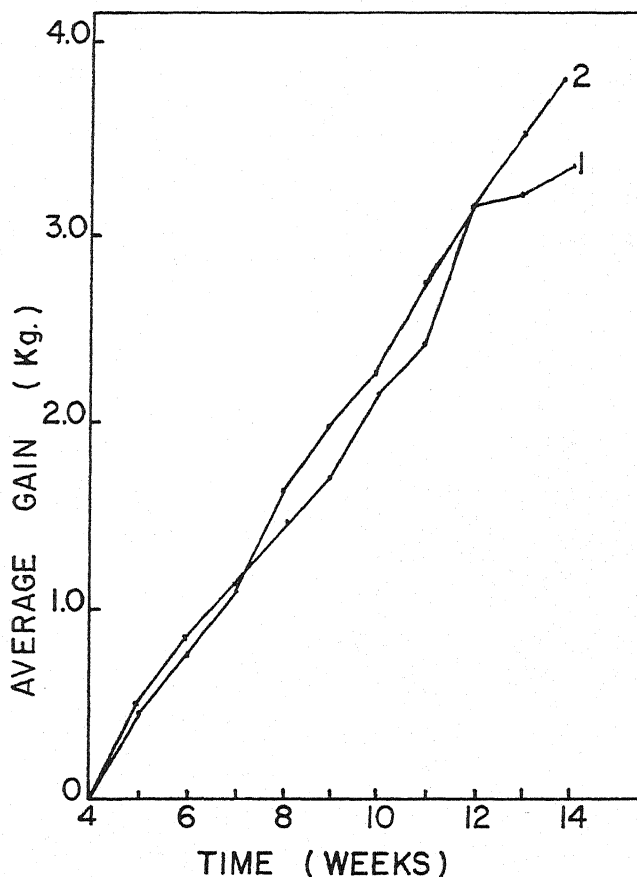


Fig. 1 Average rate of gain of dogs fed the commercial ration (curve 1) or the commercial ration plus 6% fat (curve 2) in experiment 1.

receiving 6% added fat, as is indicated in table 3. Therefore, the rates of gain per week for these two groups are shown as a single curve in figure 2. No differences in general appearance, health, and haircoats were noticed in any of the groups tested.

Organoleptic evaluations of the rations with added fat showed that no detectable rancidity developed during storage for two months at room temperature.

The average gain per week for the various groups used in the second experiment are summarized in figure 3 and the

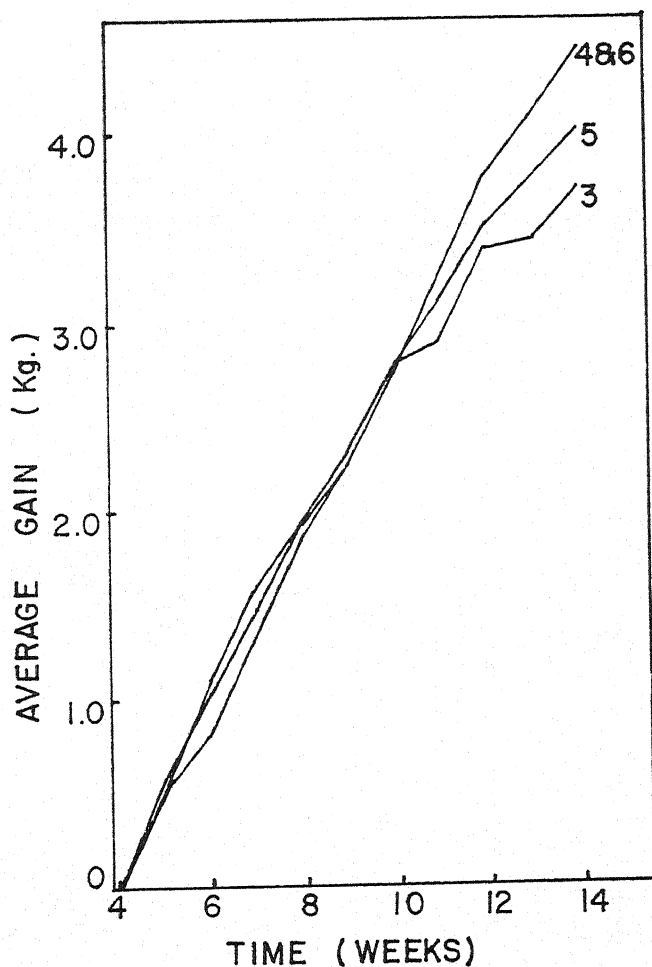


Fig. 2 Average gain per week for dogs fed the basal ration (curve 3) or this ration + 6% fat (composite for calorie-restricted and ad libitum-fed groups, curves 4 and 6—see text) or the basal ration + 13.5% sucrose (curve 5), experiment 1.

results over a 10-week period for the food and caloric efficiencies are shown in table 3. The results for this experiment confirmed those obtained in the first experiment, in

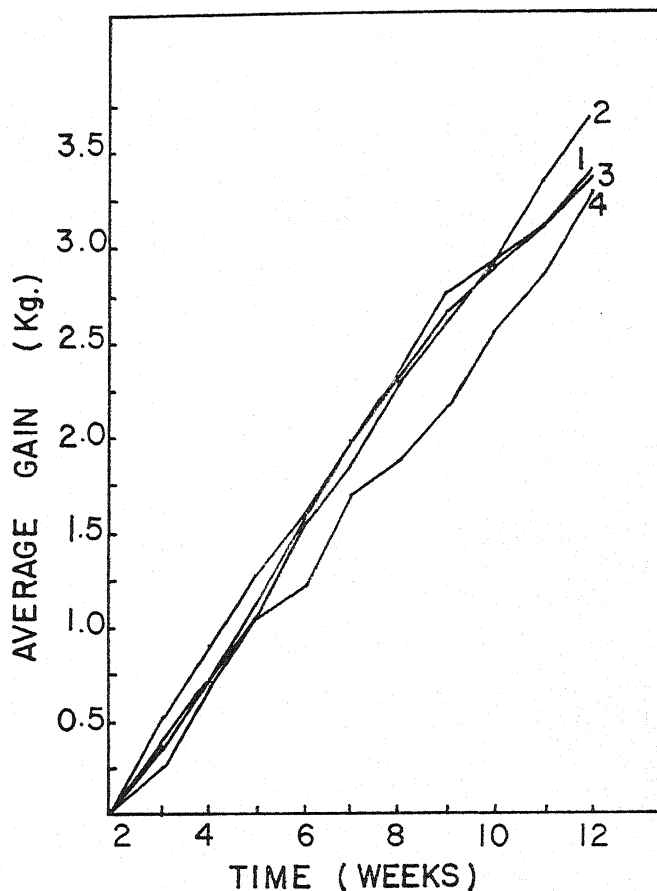


Fig. 3 Average gain for the groups fed the basal diet (curve 1), basal diet + 4% fat (curve 2), basal diet + 8% fat (curve 3), or basal diet + 18% sucrose (curve 4) in the second experiment.

that the average gains per week and the food efficiencies of all the groups tested were quite uniform, although the group receiving 4% added fat showed somewhat higher average gains per week.

Somewhat lower rates of gain were observed in the second experiment than in the first experiment. This variation may have been due to the absence of male dogs and the smaller body conformation of many of the dogs used in this second experiment. The caloric efficiencies for the groups fed added fat or sucrose were somewhat lower than for the basal group in this experiment. Except for the two dogs excluded from the results, the general appearance, haircoat and health of all dogs were excellent.

The results on an over-all basis are in good agreement for the two experiments and indicate that the performance of weanling cocker spaniel pups fed the basal diet plus 4, 6, or 8% fat was equal to or slightly better than that of the dogs fed the basal diets without added fat, based on rates of gain, general appearance, food utilization and the health of the pups.

These results indicate that widening the protein, mineral, and vitamin-to-calorie ratios by the addition of fat did not reduce the rate of gain. Therefore, the protein, mineral and vitamin content of the experimental ration was in excess of the requirements of cocker spaniels during the stage of growth used in these studies.

SUMMARY

The rate of gain of young cocker spaniel pups fed diets comprised of ingredients commonly used in dry meals, with or without added fats (choice, white grease stabilized with anti-oxidants), was investigated. The rates of gain for a 10-week period, when 4, 6, or 8% fat was added to the basal diet or when 6% fat was added to a commercial meal, were equal or slightly superior to those obtained when the diets without added fat were fed. No significant differences in the food or caloric efficiencies were noted between the groups fed different levels of fat, which indicated that the calories from the fat were well utilized. The performance of groups fed sucrose (equivalent in crude calories to the added fat) was comparable to that of groups fed the basal diet.

It is concluded that 4, 6, or 8% stabilized fat can be successfully added to the experimental ration used in these experiments, as judged by rates of gain, food utilization and the general health of young cocker spaniel pups.

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THE INFLUENCE OF DIETARY FAT AND CARBOHYDRATE ON REPRODUCTION AND LACTATION IN RATS¹

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The value of dietary fat for successful reproduction and lactation has been established for both natural and purified diets with a number of species (Evans et al., '34; Maynard and Rasmussen, '42; Nelson and Evans, '47a). The latter reported that although growth and reproduction could be satisfactorily maintained in long-term studies with purified diets, the function of lactation involves additional dietary requirements. These workers (Nelson and Evans, '47b) found an inverse relationship between lactation performance and the fat content of the diet that could be corrected by supplementation with liver eluate powder. Sica and Cerecedo ('48), however, maintained that a diet which is adequate in all respects for reproduction will also be adequate for lactation, and proposed the birth weight of the young as the primary criterion for evaluating a diet.

Deuel and co-workers ('47, '50) have more recently investigated the influence of varying amounts and kinds of fat in purified diets. Here the results were less conclusive, indicating that the type of fat in itself exerted little influence on lactation as long as it was present in appreciable amounts. Meyer et al. ('51), however, in a continuation of studies concerning the impairment of fertility and lactation by diets containing pork, were unable to assign a definite

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reason for such an effect. They emphasized the need for basic investigation concerning the influence of the composition of the diet as a whole on the requirements for reproduction and lactation. They found that reduction in the fat content of their pork-containing diets, either by adding sucrose (dilution effect) or by removal of a portion of the fat, resulted in an improvement in fertility and lactation. This finding appears significant in view of evidence to be presented in this paper.

A series of experiments summarized by Swift and Black ('49) revealed the beneficial effects of dietary fat in the reduction of heat production and heat increment and in the increased efficiency of food utilization, producing greater growth with high-fat diets as compared to high-carbohydrate diets of equal energy content. Since these experiments involved specific periods in the lifetime of the rat, it was deemed desirable to extend the work to include comparable observations on reproduction and lactation, which are discussed in the present report, and growth and longevity studies still in progress.

EXPERIMENTAL

The three diets shown in table 1 were prepared at monthly intervals, sampled for analysis, and stored in a refrigerator until fed. The control diet consisted of a finely ground commercial rat ration that had given satisfactory reproduction and lactation for a number of years in the breeding colony.² The two experimental diets, one high in carbohydrate and one high in fat, were prepared by mixing the control diet at an 80% level with 20% of corn oil or 20% of sucrose, respectively. The latter two diets were supplemented equally with liver powder, yeast and vitamins to insure optimum levels of known and unknown factors required for reproduction and lactation.

The experimental feeding extended over a period of more than two years. Since it would be impractical to store one

² Rockland Farms Rat Diet, complete (*see bottom of next page*).

lot of feed for such a time, each monthly mixing included all three diets prepared from one batch of the commercial ration. Successive monthly mixes utilized different lots of this ration but were fed to similar numbers of litter-mate animals distributed evenly among the three diets. In this manner any minor change in the composition of the commercial ration as the experiment progressed would influence equally each of the three diet groups.

The experimental animals were obtained by breeding closely related albino rats of the Wistar strain that had reached maturity on the experimental diets as a part of the growth and longevity studies. Approximately 20 males and 20 females were chosen from each of the three diet groups and were mated individually when they reached 120 days of age to produce the first generation of young. (First-litter young of this strain have been observed to be the equal of young from subsequent litters when the dams are allowed to mature to 120 days of age and 230 gm weight before mating.)

Succeeding generations were obtained by retaining a male and female from each litter, feeding the same experimental

ROCKLAND FARMS RAT DIET				
<i>Mineral composition</i>				
(By chemical determination, %)				
Silica (Si O ₂)	0.59	Calcium (Ca)		2.13
Chlorine (Cl)	0.96	Phosphorus (P)		0.71
Sodium (Na)	0.88	Sulphur (S)		0.24
Potassium (K)	1.12	Magnesium (Mg)		0.33
Milligrams per 100 gm				
Manganese (Mn)	6.80	Boron (B)		1.37
Iron (Fe)	37.00	Cobalt (Co)		0.35
Zinc (Zn)	2.00	Iodine (I)		1.20
Copper (Cu)	0.27	Fluorine (F)		1.90
<i>Vitamin content</i>				
(By rat bioassay determination, per 100 gm)				
Vitamin A	610-680 I.U.	Inositol (free) ^{a b}		61 mg
Vitamin B ₁	228-465 µg	Vitamin D	148 U.S.P. units	
Riboflavin B ₂	538-652 µg	Alpha-tocopherol		7 mg
Pyridoxine B ₆	209-275 µg	PABA ^b		78 µg
Choline equiv.	480-492 µg	Pantothenic acid	1989-2568 µg	
^a Mouse bioassay.				
^b Also by microbiological assay.				
(By chemical determination, per 100 gm)				
Niacin	14.4 mg	Carotene		42-45 mg
Vitamin C	3.60 mg	Choline		152 mg
Ratios				
Ca:P	3:1	Mn:B ₁		14:1
Ca:Mg	6.5:1	Na:K		1:1.3
anti-alopecia				
inositol diet level				
Purified 70% Stock 30%				
Guaranteed analysis				
Protein	21.00%	Fat	4.00%	Fibre 6.00%

diets until they reached 120 days of age, and then breeding them with mates from different litters but receiving the same diets. In the third generation the females were rested one month after weaning their young and then rebred to different males to obtain a second litter. Since the data showed no increase in size, weight or number of young from the second

TABLE 1
Composition of diets

	CONTROL	CARBOHYDRATE	FAT
	<i>gm</i>	<i>gm</i>	<i>gm</i>
Constituents			
Rat ration ¹	100	80	80
Sucrose	..	20	..
Corn oil ²	20
Supplements			
(per 100 gm diet)			
Liver, 1-20, ³ gm	..	1.0	1.0
Yeast, brewers', gm	..	0.5	0.5
Yeast, irradiated, gm	..	0.5	0.5
Choline chloride, mg	..	160	160
Alpha-tocopherol acetate, mg	..	5	5
Thiamine hydrochloride, mg	..	0.2	0.2
Analysis			
Energy, Cal./gm	3.910	3.935	5.011
Nitrogen, %	3.77	3.09	3.11
Ether extract, %	4.35	3.38	22.72
Moisture, %	10.33	8.23	7.87

¹ Rockland Farms Rat Diet, complete.

² Mazola.

³ Wilson's 1-20 liver powder.

breeding, these results were averaged with those of the first breeding for this generation. An average of 78 matings for each diet in three generations was thus completed. When pregnant, the females were removed from the screen-bottomed cages and placed in individual cages with wood shavings for nesting. The young were counted, examined for sex, and weighed soon after birth and at three days of age, at which time each litter was reduced to 6 rats, preferably three of

each sex as recommended by Daggs ('35). They were weighed again at 14 days of age and when weaned 21 days after birth. Approximately 350 young in the three generations were raised on each diet. The dams were similarly weighed after parturition, and at three days, 14 days and 21 days. The environmental temperature was maintained throughout the experiment at $27 \pm 2^\circ\text{C}$. with few exceptions.

Food was supplied ad libitum, and although food consumption was determined in the growth and longevity phases, spillage and contamination with wood shavings and excreta in the nesting cages prevented it in the present study. The average daily consumption of the fat, carbohydrate, and control diets by non-pregnant females of like age, weight and breeding as measured in the growth studies was 53.6, 54.7 and 59.8 Cal., respectively.

The methods used for analysis of the composited diet samples for energy, nitrogen, crude fat and moisture were, respectively, the bomb calorimeter, Kjeldahl procedure, Soxhlet ether extraction, and vacuum drying to constant weight over concentrated sulfuric acid.

RESULTS AND DISCUSSION

Since the reproduction and lactation performances compared in this experiment were determined on essentially complete and natural diets where differences were expected to be minimum, every precaution was taken to record all data that might indicate any slight effect. Accordingly, the techniques and measurements proposed by Daggs ('35), Schweigert ('47), Sica and Cerecedo ('48), Mirone et al. ('48), Henderson et al. ('48), Goettsch ('49), Sherman et al. ('49) and others were considered and as many as possible adopted.

Reproduction

If we may define good reproductive performance as the ability to conceive and give birth to large litters of living young of sufficient birth weight to insure survival, then it

will be observed (table 2) that the animals receiving the high carbohydrate diet were in all respects similar to those on the control diet. The rats in the high-fat group, however, in each generation produced fewer numbers of young per litter and young of lighter weight. This result appears even more significant when it has been observed that under normal conditions smaller litters generally consist of heavier-than-average young. While it is true that the protein levels in the fat and carbohydrate diets were somewhat lower than in the control diet (19.4, 19.4 and 23.6%, respectively), the improved quality of the protein from the yeast and liver supplements would tend to minimize this difference. The overall reduction in protein intake on the two experimental diets was not severe enough to limit growth or lactation and it seems unlikely that it contributed to the decreased reproductive performance of the high-fat group, although this possibility cannot be excluded. Certainly the slight differences in caloric intake between the fat and carbohydrate groups would not have been responsible for the significant difference in reproductive performance.

The female young at birth on each diet weighed about $\frac{1}{2}$ gm less than the males and the possibility existed that a shift in sex ratio of the young might have contributed to the apparent dietary effect. This was not found to be the case, however. The numerical ratios of male to female young born were found to be 1.06, 1.01 and 1.02 for the control, carbohydrate and fat diets, respectively. Although the numbers involved were not large, it would appear that the sex ratio was not affected by diet and did not contribute to the differences in birth weight observed between the diets.

The average birth weight of the young in all groups exceeded the minimum range for survival of 5.0 to 5.4 gm (Sica and Cerecedo, '48) and no differences were observed in the number of young surviving to the third day. All three diets would appear to be adequate with respect to specific dietary factors required for reproduction.

TABLE 2
Summary of reproduction data

DIET	GENERA- TION	NUMBER OF			NUMBER OF YOUNG BORN			AVERAGE BIRTH WEIGHT OF YOUNG	
		Dams	Matings	Result in litters ¹	Dead	Alive	Alive on 3rd day	Male	Female
Control	1st	17	17	15	2	173	171	6.4	5.9
	2nd	19	19	16	8	144	140	6.6	6.1
	3rd	14	28	22	5	214	209	6.5	6.0
	Total	50	64	53	15	531	520		
	Average per litter				0.3	10.0 ± 0.44 ²	9.8 ± 0.44	6.5 ± 0.08	6.0 ± 0.07
Carbo- hydrate	1st	21	21	21	16	206	206	6.5	6.0
	2nd	21	21	19	5	209	209	6.3	5.9
	3rd	18	36	30	14	296	286	6.5	6.1
	Total	60	78	70	35	711	701		
	Average per litter				0.5	10.2 ± 0.37	10.0 ± 0.38	6.4 ± 0.07	6.0 ± 0.07
Fat	1st	23	23	21	12	199	198	6.2	5.8
	2nd	23	23	23	14	203	195	6.3	5.8
	3rd	22	45	43	29	406	391	6.0	5.6
	Total	68	91	87	55	808	784		
	Average per litter				0.6	9.3 ³ ± 0.32	9.0 ³ ± 0.33	6.1 ⁴ ± 0.08	5.7 ⁴ ± 0.07

¹ All unsuccessful matings involved two or more trials with fertile males.

² Standard error of the mean.

³ Significantly different at 5% level from carbohydrate group.

⁴ Significantly different at 1% level from either control or carbohydrate group.

A somewhat higher proportion of matings were successful in animals receiving the high-fat as compared to the other diets, but when the failures were examined individually, similar numbers of resorptions were noted; 4, 4, and three, respectively, for the control, carbohydrate and fat diets. One female in each of the carbohydrate and fat groups died at parturition and the few remaining failures were considered to be due to female sterility after two or more unsuccessful matings with fertile males.

The average number of days elapsing between mating (as defined by placing a male and female together) and parturition was similar in all diet groups, averaging 27.2, 28.0 and 27.7 days for rats on the control, carbohydrate and fat diets, respectively. The majority of the females gave birth 23 to 28 days after mating, with a few on each diet, however, requiring as many as 50 days. Male fertility was excellent in all diet groups through all generations.

Lactation

Lactation performance was measured primarily by recording the gain in weight of the young during the period from the third to the 14th day, during the time that their sole source of nourishment was obtained by nursing. The data, summarized in table 3, indicate a significant beneficial effect on lactation of the high-carbohydrate diet. The young of each sex in the carbohydrate group gained about 7% more weight than did those on the fat or control diets. This finding extended into the third week of lactation, when the young were gradually consuming increasing amounts of the experimental diets. The average gain in weight during the third week was 14.7 and 13.9, 17.5 and 16.7, and 15.3 and 15.2 gm for the males and females in the control, carbohydrate and fat groups, respectively. At 21 days of age the young of both sexes on the carbohydrate diet were significantly heavier than the other two groups. The difference was in the order of 10%, indicating that the improvement in lactation was ef-

TABLE 3

Summary of lactation data

DIET	GENERATION	NUMBER OF YOUNG ¹	AVERAGE GAIN IN WEIGHT FROM 3RD TO 14TH DAY OF AGE		AVERAGE WEANING WEIGHT AT 21 DAYS OF AGE	
			Male	Female	Male	Female
Control	1st	90	gm 20.6	gm 19.8	gm 44.3	gm 42.1
	2nd	66	21.9	21.1	46.4	43.9
	3rd	108	19.1	18.9	43.5	41.7
	Total Average	264	20.3 ± 0.43 ²	19.8 ± 0.39	44.5 ± 0.65	42.4 ± 0.60
Carbohydrate	1st	102	22.6	21.5	49.2	47.1
	2nd	108	21.6	20.9	48.3	46.6
	3rd	156	21.9	21.0	49.0	46.5
	Total Average	366	22.0 ³ ± 0.30	21.1 ³ ± 0.29	48.8 ³ ± 0.55	46.7 ³ ± 0.52
Fat	1st	108	22.0	21.5	47.6	46.5
	2nd	120	19.8	19.2	44.1	42.7
	3rd	216	19.8	19.2	43.4	42.4
	Total Average	444	20.3 ± 0.29	19.8 ± 0.32	44.6 ± 0.45	43.5 ± 0.48

¹ All litters reduced at three days of age to 6 young, usually three of each sex.² Standard error of the mean.³ Significantly different at 1% level from either control or fat group.

fective in producing heavier weanling animals. A similar beneficial effect of sucrose was observed by Meyer et al. ('51).

It is interesting to note that the high-fat diet was the equal of the control diet in promoting lactation, in contrast to its limiting effect on reproduction. Further, the improvement in lactation resulting from the high-carbohydrate diet as compared to the control diet was not accompanied by any beneficial effect on reproduction. These observations support the thesis of Sica and Cerecedo ('48) that the reproductive process is the more critical of the two.

All dams were successful in rearing the 6 young given them to nurse with the exception of one on the fat diet that lost one of her young between the 14th and 21st days of lactation.

The average change in weight of the dams during the lactation period from the third to the 14th day was + 7.0, + 9.4 and - 4.2 gm, respectively, for the control, carbohydrate and fat diets, and from the 14th to 21st day these values were - 10.0, - 8.0 and - 2.2 gm. The individual variation on all diets was extreme, however, ranging from + 49 to - 34 gm. A more severe loss in weight of dams receiving high-fat diets during the first 17 days of lactation was reported by Maynard and Rasmussen ('42) in paired feeding studies involving isocaloric intakes at different fat levels of diets composed of either natural or purified components. The weight loss was reversed, however, when the high-fat, purified diet was fed *ad libitum*. There is some question whether this result would have been obtained if all factors necessary for lactation had been provided in optimum amounts or if the diet which contained 18% fat had consisted of natural foods. Purified diets containing up to 40% of fat have been shown by Deuel et al. ('47) to permit excellent reproduction and lactation.

Maynard and Rasmussen also found a fat level of 9% superior to one of 4.5% in natural food diets, whereas in the present experiment a diet containing 23% of fat was just equal to the control diet (4.4% fat) and was less efficient than the high-carbohydrate diet (3.4% fat) in promoting lac-

tation. Apparently the optimum level of fat may be greatly influenced by the composition (purified or natural food components) of the diet.

SUMMARY

Reproduction and lactation performance was measured for three generations with albino rats fed diets ad libitum composed essentially of natural foods and varying in fat and carbohydrate content.

With an average number of 78 matings for each diet, a significant decrease in reproductive performance of rats receiving the 23% fat diet was observed when compared to the control (4.4% fat) and the high-carbohydrate-fed (3.4%) animals. This was manifest in smaller numbers of lighter weight young in the litters of the high-fat group.

Lactation performance, measured with an average number of 350 nursing young for each diet, revealed no impairment due to the high-fat diet as compared to the control diet. However, a significant improvement in lactation resulted from the high-carbohydrate diet which contained sucrose. Weanling weights and weight gains of the young during the critical lactation period were increased 7 to 10% in this group.

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NITROGEN CONSTITUENTS OF SOW'S MILK AS AFFECTED BY RATION AND STAGE OF LACTATION¹

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The literature reveals very little information on the detailed composition of sow's milk. The main reasons for this lack of information are the difficulties involved in obtaining representative samples in large enough quantities for analysis. Various workers have reported studies on gross composition. The majority of the reports were not concerned with the effects of either ration or stage of lactation, but merely with composition per se. This, to a large extent, helps to explain the wide disparity in the reported data.

More recently Braude et al. ('46, '47) reported on the gross composition of sow colostrum and normal milk, their work including analyses for several vitamins. Bowland et al. ('49a, b, c) studied the effect of nutrition on the gross composition of sow's milk, comparing stage of lactation and dry-lot versus pasture feeding primarily with respect to content of vitamins A and C. The studies of both Braude and Bowland were conducted under controlled conditions.

None of the aforementioned studies includes data on the various nitrogenous components of sow's milk.

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EXPERIMENTAL PROCEDURE

The work reported herein will be discussed in 4 different sections.

Section I

Twenty-five sows, 8 Chester Whites and 17 Poland Chinas, were distributed at random into 5 lots. From weaning until two months before breeding these sows were fed a ration of corn, soybean oil meal, and 5% alfalfa. During the course

TABLE 1
Ration of sows in section I¹

INGREDIENTS OF RATION	LOT				
	I	II	III	IV	V
	<i>lb.</i>	<i>lb.</i>	<i>lb.</i>	<i>lb.</i>	<i>lb.</i>
Yellow corn	79.0	80.0	81.0	83.5	79.0
Soybean oil meal	14.5	8.5	7.5	..	14.5
Ground alfalfa	5.0	5.0	5.0	5.0	5.0
CaCO ₃	1.0	1.0	1.0	1.0	1.0
Iodized salt	0.5	0.5	0.5	0.5	0.5
Fish solubles	..	5.0
Tankage	5.0	10.0	..
B ₁₂ concentrate	0.5

¹ All rations adjusted to an equivalent total protein content (14%).

of the experiment the sows were fed as indicated in table 1. Samples of colostrum,³ 15-, and 30-day milk were analyzed for total and casein nitrogen. The milk was extracted manually after injection of 1 ml of Pitocin⁴ into the ear vein of the sow. Two men, one on each side, milked simultaneously as rapidly as possible in order to complete the milking by 6 minutes after the oxytocin injection. Four hundred to 600 gm per milking were obtained. All teats were milked. The results are summarized in tables 2 and 3.

³ In this work colostrum is milk extracted 12 to 24 hours after parturition.

⁴ Parke-Davis.

TABLE 2

Effect of ration on the distribution of the nitrogen of colostrum, 15-, and 30-day milk of sows in section I

ITEMS COMPARED	LOT				
	I	II	III	IV	V
Total nitrogen (mg per 100 gm)					
Colostrum	1,354 ± 94.4 ¹	1,501 ± 131.2	1,827 ± 209.6	1,720 ± 99.2	1,694 ± 224.0
15-day milk	.. ²	824 ± 44.8	757 ± 43.2	763 ± 24.0	819 ± 8.0
30-day milk	.. ²	994 ± 116.8	869 ± 52.8	768 ± 25.6	813 ± 22.4
Casein nitrogen (mg per 100 gm)					
Colostrum	671 ± 75.2	678 ± 17.6	611 ± 36.8	752 ± 28.8	725 ± 84.8
15-day milk	.. ²	533 ± 17.6	550 ± 44.8	546 ± 69.6	557 ± 56.0
30-day milk	.. ²	605 ± 24.8	546 ± 11.2	544 ± 68.8	558 ± 19.2

¹ Standard error of the mean.

² All of the baby pigs died and the sows ceased lactating before the 15th day.

TABLE 3

Effect of stage of lactation on the distribution of the nitrogen of colostrum, 15-, and 30-day milk of sows in section I

ITEMS COMPARED	COLOSTRUM	15-DAY MILK	30-DAY MILK
Total nitrogen (mg/100 gm)	1646 ¹ ± 76.8 ²	808 ± 27.2	862 ± 40.0
Total protein (%)	10.50 ± 0.49	5.15 ± 0.17	5.50 ± 0.26
Casein nitrogen (mg/100 gm)	683 ¹ ± 24.0 (41.5) ³	554 ± 28.8 (68.6) ³	565 ± 28.8 (65.5) ³
Casein protein (%)	4.35 ± 0.15	3.53 ± 0.18	3.60 ± 0.29
Other nitrogen (mg/100 gm)	963 ¹	254	298
Other protein (%)	6.15 (58.5) ³	1.59 (31.4) ³	1.90 (34.5) ³

¹ Significant at 1% level.

² Standard error of the mean.

³ Percentage of the total protein.

Section II

Three sows selected at random from section I in their 7th week of lactation were milked. Sow 2 came from lot V and the other two sows were from lot IV. Their milks were analyzed in great detail, with special emphasis on the nitrogenous fractions. The results are tabulated in table 4.

TABLE 4
Detailed analysis of sow's milk
(mg per 100 gm milk)

N-FRACTION	SOW			Average
	2	4	7	
Total N	1,126	992	880	999
Casein N	693	545	477	572
Albumin N	93.1	62.4	67.5	74.3
Globulin N	97.5	112.5	95.8	101.9
Proteoses-peptone N	164	188	175	176
N.P.N.	78.3	84.0	64.8	75.7
Ammonia N	0.80	1.75	0.95	1.16
Urea N	12.3	13.2	13.7	13.1
Creatinine ¹	1.90	1.78	1.80	1.83
Creatine ¹	9.65	10.8	7.40	9.28
Uric acid ¹	2.84	2.20	2.03	2.36
Alpha-amino N	27.8	18.8	22.2	22.9
N unaccounted for	32.6	45.3	24.2	34.0
Total solids, %	21.0	19.2	17.4	19.2
Fat, %	8.31	7.46	5.62	7.13
Lactose, %	5.07	6.76	5.31	5.71
Sulfur, mg %	64.2	73.3	63.7	67.1
pH	7.00	7.07	6.90	6.99
Specific gravity	1.036	1.040	1.039	1.038
Freezing point, °C.	-0.562	-0.564	-0.564	-0.563

¹ Reported as such. N unaccounted for calculated on the basis of N only.

Section III

Twelve grade Hampshire sows, 6 between the 4th and 15th day of lactation and 6 between the 15th and 38th day of lactation, were milked, and analyses for total, casein, and non-protein nitrogen were made. Table 5, part A, presents a summary of these data. These sows were on excellent ladino-

alfalfa pasture during the gestation period. During the last half of the gestation period, in addition to pasture, they were hand-fed first one and then two ears of corn per sow per day. Seven days prior to farrowing they were taken to the farrowing barn and self-fed the following ration: 15 parts alfalfa, 15 parts of a 40% protein commercial hog supplement, 5 parts of corn, 15 parts of wheat, 48 parts of oats, and one part each of CaCO_3 and trace mineralized salt (16.3% total protein).

Section IV

Four purebred Poland China and 6 purebred Chester White sows were milked on the first, 15th, and 30th day. The samples were frozen and analyzed for non-protein nitrogen three months later. Prior to farrowing these sows were on good alfalfa-brome-ladino pasture and were self-fed soft ear corn in addition to a mixture consisting of 46 parts ground corn, 30 parts oats, 17 parts wheat middlings, three parts linseed meal, three parts tankage, and a half part each of calcium carbonate and trace mineralized salt (13.3% total protein). Three days before farrowing the sows were brought into the farrowing barn and hand-fed the following mixture: 20 parts ground corn, 20 parts oats, 20 parts wheat middlings, 15 parts wheat bran, 8 parts linseed meal, 4 parts tankage, one-half part calcium carbonate, one-half part trace mineralized salt, and 12 parts alfalfa leaf meal (17.3% total protein). The sows were fed a limited quantity for a few days after farrowing and then full-fed the above mixture. The summary of this study appears in part B of table 5.

All the methods for determining protein and non-protein fractions standardized for cow's milk (Shahani and Sommer, '51) were applicable to sow's milk, except that smaller aliquots were taken for the total, non-protein, globulin, and alpha-amino nitrogen determinations. Total solids were determined by drying a weighed sample of milk in a vacuum drying oven, at 70°C. and 5 mm vacuum. Fat was determined by the Mojonnier technique, lactose by the Munson

and Walker copper reduction method, and sulfur by reducing sulfur to hydrogen sulfide, which was estimated iodometrically. The Beckman pH meter was used for pH determinations; the Quevenne lactometer, for specific gravity; and the Hortvet cryoscope, for the freezing point determinations.

TABLE 5

Lactation and nitrogenous constituents of milk

Part A. Effect of stage of lactation

ITEMS COMPARED	4-15 DAY	15-38 DAY
Total nitrogen (mg/100 gm)	780 \pm 34.2	808 \pm 45.5
Total protein (%)	4.98 \pm 0.22	5.16 \pm 0.29
Casein nitrogen (mg/100 gm)	495 \pm 24.5	506 \pm 21.9
Casein protein (%)	3.16 \pm 0.16 (63.5) ¹	3.23 \pm 0.14 (62.6) ¹
Non-protein nitrogen (mg/100 gm)	83.1 \pm 8.5	95.4 \pm 10.4
(N \times 6.38) % protein	0.53 \pm 0.05 (10.6) ¹	0.61 \pm 0.07 (11.8) ¹
Other nitrogen	202	207
(N \times 6.38) % protein	1.29 (25.9) ¹	1.32 (25.6) ¹

Part B. Effect of lactation on N.P.N. of sow's milk

ITEMS COMPARED	1-DAY	15-DAY	30-DAY
Non-protein nitrogen (mg/100 gm)	70.8 \pm 3.9	131 \pm 8.1 ²	137 \pm 6.8 ²
Expressed as protein (%)	0.45 \pm 0.02	0.84 \pm 0.05	0.88 \pm 0.04

¹ Percentage of the total protein.

² Significant at 1% level.

RESULTS

Section I

The object of this experiment was to determine the effect of vitamin B₁₂ and animal proteins upon the various nitrogenous constituents of the milk of sows fed a corn, soybean oil meal, 5% alfalfa (C.S.A.) ration. This ration has been shown by Rose et al. ('44) to cause failure in both reproduction and lactation. It has been suggested that the milk of these sows may lack certain qualities possessed by the milk of sows fed rations supplemented with animal proteins.

Since vitamin B₁₂ has been associated with the metabolism of protein (Charkey, Wilgus, Patton and Gassner, '50; Hart-

man, Dryden and Cary, '49; Henry and Kon, '51), one would look for differences in the nitrogenous components of milk from sows on C.S.A. rations. Although considerable variation within lots was observed, colostrum, 15-, and 30-day milk from sows in all groups had a similar total nitrogen and casein nitrogen content (table 2). The total nitrogen values for the colostrum of the sows in lot I tended to be lower than those for the colostrum of sows fed B₁₂ or animal protein, but these differences were not significant (F test). The total nitrogen content of 15- and 30-day milk was not significantly different in the B₁₂- or animal protein-supplemented lots. The casein nitrogen tended to be less variable than the total nitrogen content.

Because there were no significant differences between treatments, it was decided to pool all the data in evaluating the effect of stage of lactation (table 3). The total protein content of the 15th day milk was less than half that for colostrum. There was a tendency for the total protein to rise slightly from the 15th day milk to that of the 30th day. This is in general agreement with the work of Bowland et al. ('49c) insofar as the trend is concerned. The levels of total protein, however, were consistently lower, and were similar to those of Braude et al. ('47).

The casein fraction was quite similar in concentration pattern to that for total protein, but the changes were not as marked. No consistent breed difference was observed.

It is of interest to note that the casein in normal sow's milk makes up only 67% of the total protein (colostrum, 41.5%), in contrast to an average of 76% in normal cow's milk (Davies, '33).

Failure to observe a difference in total or casein protein (table 2) is not surprising, since it is generally known that when the lactating animal is forced to conserve milk, because of a scarcity of structural units, it lowers the flow, but at the same time tends to maintain the milk's normal composition. Thus, with the exceptions of fat (Willett and Maruyama, '46), vitamins, and the various trace minerals, the or-

ganic and inorganic constituents of milk remain relatively constant under wide variations of diet. Parrish et al. ('48), feeding high and low protein rations to dairy cows, reported that the rations did not effect significant differences in the levels of the total protein, casein, and albumin-globulin fractions of colostrum or early milk. Riddet et al. ('41) were able to show a definite decrease in the non-fat-solids of cow's milk when cows were under conditions of subnormal feeding. This was ascribed to a decrease of approximately 0.2% in total protein and an approximate 0.1% decrease in lactose content. Regan and Richardson ('38) made a similar observation, but only after subjecting the cows to high atmospheric temperatures.

Section II

Sow's milk was found to be richer, greater in specific gravity, more alkaline in nature, and to have a slightly lower freezing point than cow's milk.

The data are in harmony with the values reported by Braude et al. ('47) except in the case of lactose. As compared to the figures cited by Jackson ('50), the values obtained in this study were higher in total solids, fat, and lactose, but lower in total proteins. A detailed analysis of the various nitrogen fractions appears in table 4. Casein accounted for 57 to 58%, albumin 7 to 8%, globulin 10%, proteoses-peptones 17 to 18%, and non-protein nitrogen 7 to 8% of the total nitrogen. Among non-protein nitrogen fractions, alpha-amino and urea nitrogen fractions were highest and both together accounted for about 85% of the measurable non-protein nitrogen. As compared to cow's milk, sow's milk was about 99% higher in total nitrogen (considering 500 mg total nitrogen per 100 ml cow's milk as average). There also was a pronounced difference between the albumin and globulin content of sow's milk and that of cow's milk. The former contained larger amounts of albumin and globulin, and the amount of globulin was larger than that of albumin, whereas in the case of cow's milk the quantity of albumin nitrogen

is greater. A similar globulin-albumin relationship exists in the plasma proteins of swine as was found in the milk.

The non-protein nitrogen content of sow's milk is significantly higher than that of cow's milk. The average non-protein nitrogen content of the three sow's milk samples was about 2.3 to 2.4 times the average for cow's milk (Shahani and Sommer, '51). Block and Bolling ('50) reported that human breast milk contained 4 times the non-protein nitrogen of cow's milk. In terms of non-protein nitrogen fractions, these higher values for sow's milk were due chiefly to the higher values found for urea, alpha-amino nitrogen, and nitrogen unaccounted for.

Section III

The same trend, a tendency for the various nitrogenous constituents of normal milk to increase with stage of lactation, was in evidence in this group of pasture-fed sows (table 5, part A). The observation by Bowland et al. ('49c) that the total protein of milk from pasture-fed sows was consistently lower than that of dry-lot sows, although the difference was not statistically significant, was borne out by this study. This work showed that these differences were due primarily to the lower level of casein in the milk of pasture-fed sow. The nitrogen other than casein was actually slightly higher in pasture-fed sows than in dry-lot sows, which was probably due to the non-protein nitrogen. One cannot say with any certainty that these differences were due to the effects of pasture versus dry-lot feeding. Although no apparent breed difference was observed in the section I study or in the work of Bowland et al. ('49c), one can never completely rule it out, particularly under slightly different interacting conditions.

Section IV

The non-protein nitrogen level of colostrum was found to be 70.8 ± 3.9 mg per 100 gm of sample, rising to almost double that value by the 15th day, with a further slight in-

crease in 30-day milk (table 5, part B). These increases over the values for colostrum were highly significant.

In studies with dairy cattle Parrish et al. ('48) were able to show that a high protein ration raised not only the non-protein nitrogen content of colostrum and normal milk but also that of blood serum, in contrast to the effect of a low protein ration. As lactation progressed there was a gradual decrease in the amount of non-protein nitrogen in the milk of both the high and low protein lots.

Perkins et al. ('32) concluded that the differences that were found in the protein composition of cow's milk as a result of changing the amount or quality of protein in the ration were principally reflected in the non-protein nitrogen portion of the milk.

In general the work reported here was characterized by the existence of considerable individual variability among sows regardless of ration or stage of lactation.

SUMMARY AND CONCLUSIONS

The effect of ration and stage of lactation on total protein, casein, and non-protein nitrogen in the milk of the sow was studied. A detailed analysis of sow's milk with emphasis on the nitrogenous constituents was made.

The additions of B₁₂, tankage or fish solubles to an all-plant protein ration did not result in significant changes in the total protein or casein content of the colostrum of sows previously maintained on a corn, soybean oil meal, 5% alfalfa (C.S.A.) ration, when the total protein of the ration was the same. Similarly, the source of protein had no effect on the amount of total or casein protein in the 15- and 30-day milk of sows in lots II, III, IV, and V.

The total protein content dropped sharply from colostrum to 15-day milk, but rose slightly in 30-day milk. The casein content values behaved similarly but the changes were not as marked. The decrease from colostrum to 15-day milk was highly significant in both instances.

The non-protein nitrogen increased significantly from colostrum to 15-day milk and there was a further increase in 30-day milk.

The mean values of sow's milk constituents (7th week of lactation) were as follows: 19.2% total solids, 7.13% fat, 5.7% lactose, 6.4% protein ($N \times 6.38$), sulfur 67.1 mg %, pH 6.99, specific gravity 1.038, and freezing point -0.563°C . Of the 6.4% protein, casein accounted for 57 to 58%, albumin 7.8%, globulin 10%, proteoses-peptones 17 to 18%, and non-protein nitrogen 7 to 8%. Alpha amino and urea nitrogen fractions made up about 85% of the accounted-for non-protein nitrogen.

ACKNOWLEDGMENTS

Acknowledgment is made to Mr. Leo P. Brunner for valuable assistance in collection of the samples. Thanks are also due to Mr. A. M. El-Negoumy for determining total solids, fat, and lactose, to Mr. A. M. Mostafa for determining sulfur, and to Mr. Saeed Gaballah for determining freezing points on these samples in connection with their respective research projects.

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PROCEEDINGS OF
THE SIXTEENTH ANNUAL MEETING OF THE
AMERICAN INSTITUTE OF NUTRITION

HOTEL NEW YORKER, NEW YORK CITY
APRIL 14-18, 1952

COUNCIL MEETINGS

Council Meetings were held in the Hotel New Yorker on Sunday, April 13, and Monday, April 14. Formal actions of the Council are reported in the following minutes of the business meetings.

SCIENTIFIC SESSIONS

The scientific program of the Institute consisted of 7 half-day sessions at which 80 submitted papers were presented and one half-day session devoted to a symposium on "Geriatric Nutrition." The symposium was held on Wednesday afternoon, April 16. Four papers were read by title.

BUSINESS MEETINGS

Two business meetings were held: one at 4:00 P.M. Tuesday, April 15, and one at 4:00 P.M. Thursday, April 17. The following items were considered:

The Tuesday business meeting. The meeting was called to order by President Clive M. McCay. The minutes of last year's business meetings, as published in the August, 1951, issue of the *Journal of Nutrition*, were approved.

The President appointed Dr. R. A. Gortner, Jr. and Dr. Charlotte Roderuck to serve as a Tellers' Committee for the election of officers. The ballots were transmitted to the Tellers' Committee by the Secretary.

The Council's recommendations on nominees for election to membership in the American Institute of Nutrition were

presented. It was voted to approve the Council's recommendations. The following were elected to membership:

Joseph T. Anderson
W. M. Beeson
Bertha S. Burke
Jack M. Cooperman
Theodore S. Friedemann
Marvin B. Gillis
Albert C. Groschke
Arthur M. Hartman
Carl M. Lyman
Charles D. May

James McGinnis
Joseph Meites
Robert E. Olson
Howerde E. Sauberlich
Burch H. Schneider
Max O. Schultze
Jakob A. Stekol
Norman C. Wetzel
Hilda F. Wiese
C. K. Whitehair

The report of the Treasurer, Dr. N. B. Guerrant, was submitted. The Auditing Committee, consisting of Dr. R. W. Swift and Dr. Alex Black, reported that receipts and disbursements are properly recorded in the Treasurer's books and that his report as of April 1, 1952, is substantiated by the record.

The report of Dr. W. C. Russell, representative of the American Institute of Nutrition to the Division of Biology and Agriculture and to the Food and Nutrition Board, National Research Council, was submitted.

The representatives on the Joint Committee on Nomenclature, Dr. C. A. Elvehjem and Dr. E. M. Nelson, reported no actions during the year.

The annual report of the Editor of the *Journal of Nutrition* was presented by Dr. George R. Cowgill. The report follows.

*Annual Report of the Editor of the Journal
of Nutrition*

This annual report covers the volumes of the *Journal of Nutrition* published during 1951 as well as general matters arising since the last annual meeting of the American Institute of Nutrition in Cleveland, Ohio, April 15, 1951.

Papers published and related data

Three volumes — numbers 43, 44 and 45 — were published during 1951. Each volume began with a biography. In addition, these three volumes contained 45, 47 and 49 articles, respectively, giving a total

of 144 for the year, compared with 151 for the previous year. The average number of pages per article proved to be 12.5, exactly the same as for 1950. The present arrangement with our publisher, Wistar Institute, calls for three volumes a year and the printing of 1,800 pages of scientific matter. During 1951 subscribers received a total of 1,941 pages; during the previous year 1,895 pages were printed. During the year 197 papers were submitted, of which 39, or 19.8%, were rejected. The corresponding figures for 1950 are 170 submitted and 43, or 25.3%, rejected.

Picture on front cover

In continuation of the policy adopted at the 1949 meeting of the editorial board in Detroit, the first issue of each volume carried a new picture in the oval on the front cover. A larger picture was used as a frontispiece, accompanied by a short biography. The pictures used in 1951 were those of Magendie, Beaumont and Bernard.

Personnel of editorial board

At the last annual meeting three members retired, having served their terms of 4 years. They were E. W. Crampton, O. L. Kline, and R. W. Swift. The new members elected to replace them were Alex Black, Floyd S. Daft, and Harry G. Day. For part of the year Dr. Hegsted was absent on professional work in Peru. He offered to resign because of this enforced absence but was prevailed upon to consider himself as being on a "leave of absence." On his return in June, 1951, he resumed active work as a member of the board.

Editorial problems

In my last annual report I commented rather extensively on these problems and therefore write about them only briefly here. They continue to be the same as those encountered in previous years. As part of our effort to deal properly with them I try to have an annual meeting of the editorial board at each Federation meeting. At such times editorial problems are reviewed, an exchange of ideas obtained, the actions of the editor reviewed, and any matter pertaining to the Journal and its operation considered. This is mentioned here in order to make clear to the members of the Institute that the editor tries not to be dictatorial in the discharge of his responsibilities, actively seeks the advice of the editorial board in the determination of policy, and endeavors to keep the board properly informed and in a position to render real service in the printing enterprise represented by the Journal.

The editorial office thinks it detects *some* improvement in the matter of authors' reading the instructions to contributors printed on the inside back cover of each issue. It is still evident, however, that many authors do *not* read such instructions.

General comments

It is appropriate to express here my deep appreciation of the services rendered by the individual members of the editorial board. Our thanks are also due to the staff of The Wistar Institute, which has always cooperated in solving special printing problems as far as possible in accordance with our wishes. Some issues have been delayed because of factors beyond their control. Examination of any issue with respect to the dates when papers were submitted for publication will reveal what appears on the surface to be an inexcusable delay in final publication. Examination of our office records in this connection, however, will show that such delay was not our fault but really due to the author's failing to give reasonably prompt attention to the revision of his manuscript. We have actually had some papers that were returned for revision sent back to us after as long as a year! A check shows that, *on an average*, an accepted paper appears in about $4\frac{1}{2}$ or 5 months after formal submission. In a few instances there have been long delays for which we were responsible and for which we offered our apologies. Fortunately, our records show such instances to be fewer and fewer. I wish we could avoid them entirely, but suppose that this represents an unattainable ideal.

A motion was passed expressing appreciation to Dr. Cowgill for his excellent administration of the affairs of the Journal.

President McCay reported on the actions of the Federation Executive Committee during the past year. Attention was directed to the activities of the "Animal Welfare Institute" in attempting to restrict or prevent animal experimentation in certain parts of the country. The Federation Executive Committee regards these activities as a serious menace to research in the biological sciences and is taking steps accordingly.

The proposed new Federation constitution and by-laws were discussed. Mimeographed copies were distributed to all members present for study. The Secretary stated that the proposed new constitution and by-laws embody the modifications approved at the business meeting last year. A few

other changes of less importance are also included. The document has been approved by the Federation Executive Committee after several months' careful study and the Council of the Institute. A motion to approve the new Federation constitution and by-laws was carried unanimously.

The meeting was adjourned at 5:10 P.M.

The Thursday business meeting. The meeting was called to order by President McCay at 4:00 P.M. At President McCay's request, an extension for another year of the changes in the Federation by-laws made last year at the Cleveland meeting was approved. Such an extension will become necessary only if the new Federation constitution and by-laws are not approved by the other societies of the Federation.

A motion to include the past-president as a member of the Council of the Institute for the coming year was approved. This change was suggested because the past-president of each Society is now a member of the Federation Executive Committee. A corresponding change in the constitution and by-laws of the Institute will be submitted for approval next year.

Dr. Pollack reported on the work of the Committee on the Registry of Pathology of Nutritional Diseases. A summary of the work of the Registry is to be published in the *Journal of Nutrition*.

Dr. W. H. Griffith discussed the organization of the American Board of Nutrition and its activities during the past year.

The Teller's Committee reported the results of their count of the ballots. A total of 230 ballots was cast. The following officers were elected for the year beginning July 1, 1952:

President:

Paul L. Day

Vice-President:

Conrad A. Elvehjem

Councillor:

Gladys A. Emerson

Associate Editors:

James B. Allison

Carl A. Baumann

L. C. Norris

Suggestions for the Nominating Committee:

The names of 16 members receiving 10 or more votes each were received.

A motion was passed to set the annual dues at \$1.00 per year, as recommended by the Treasurer and approved by the Council.

As approved by the Council, the recommendation that the annual \$3.00 Federation assessment per member be paid, if desired, by retired members was unanimously adopted. As is the case in other societies of the Federation, retired members of the Institute now may or may not elect to pay the assessment and receive in return the *Federation Proceedings*. In either event, they will continue to be listed in the Federation Directory. Heretofore, the assessment has been paid from the treasury of the Institute. It was pointed out that this practice could no longer be continued without an increase in Society dues, because of the increasing number of emeritus members.

The desirability of re-establishing a Committee on Food Habits and Methods of Education by the National Research Council, either as a unit or with the Food and Nutrition Board, was discussed by Dr. P. E. Howe. By motion, the members approved the establishment of such a committee and the aiding of the project in any way possible.

President McCay announced that the members of the American Institute of Nutrition have been invited to participate in the 19th International Physiological Congress to be held in Montreal, August 31 to September 4, 1953. Notices regarding the meetings will be mailed to members at a later date.

Dr. E. M. Nelson spoke briefly on the progress of the organization of the International Union of Nutritional Sciences.

President McCay announced that next year's meetings will be held in Chicago. In 1954 they will be held in Atlantic City and, tentatively, in Los Angeles in 1955. The Federation Executive Committee has requested each Society secretary to poll the membership as to preference for meeting *annually* in Atlantic City after 1955.

The meeting was turned over to the new President, Dr. Paul L. Day, and adjourned at 5:15 P.M.

THE ANNUAL DINNER AND PRESENTATION OF AWARDS

The Annual Dinner of the Institute of Nutrition was held on Wednesday evening, April 16, in the Hotel New Yorker. The program consisted of the introduction of new members, the presentation of awards, and a few informal remarks by President McCay.

The Borden Award was presented to Dr. Max Kleiber of the University of California at Davis for his contributions regarding factors involved in the formation and utilization of the constituents of cow's milk. The achievements of the medalist were described by Dr. Walter C. Russell of Rutgers University. Dr. Kleiber responded with a brief resumé of the work of his group.

The Mead Johnson Vitamin B-Complex Award was presented to Dr. Howerde E. Sauberlich of the Alabama Polytechnic Institute in recognition of his fundamental investigations on the citrovorum factor and its relation to folic acid. Dr. W. D. Salmon, of the same institution, introduced the Award recipient and commented on his contributions. Dr. Sauberlich then spoke humorously regarding his investigations.

The Osborne and Mendel Award was presented to Dr. Icie Macy Hoobler, Scientific Director of the Children's Fund of Michigan, for her outstanding studies on the nutritive requirements of children. Dr. Arthur H. Smith of Wayne University, in introducing the medalist, spoke of her early training and her many subsequent achievements in nutritional research on children. Dr. Hoobler responded with reminiscences of her studies and with a tribute to the inspiration and guidance which gave direction to her work by her teacher, for whom the award was jointly named, Professor Lafayette B. Mendel.

COMMITTEES FOR 1952-1953

President Clive M. McCay appointed the following committees for the year beginning July 1, 1952:

Nominating Committee

J. H. Roe, Chairman
R. J. Block
L. R. Cerecedo

W. D. Salmon
Pearl P. Swanson

Committee on Registry of Pathology of Nutritional Diseases

H. Pollack, Chairman
O. A. Bessey

W. H. Sebrell, Jr.
E. L. Sevringhaus

Representatives to the Joint Committee on Nomenclature

C. A. Elvehjem

E. M. Nelson

Representative to the Division of Biology and Agriculture and to the Food and Nutrition Board, National Research Council

W. C. Russell

Respectfully submitted,

JAMES M. ORTEN, *Secretary*
American Institute of Nutrition

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1953 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it will be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1953. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1953. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

Chairman, Nominating Committee:

DR. LEO T. SAMUELS

*Department of Biological Chemistry
University of Utah Medical School
Salt Lake City, Utah*

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1953 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1953.

Chairman, Nominating Committee:

DR. PHILIP HANDLER

*Department of Biochemistry and Nutrition
Duke University School of Medicine
Durham, North Carolina*

DERMATOSIS IN WEANLING RATS FED LACTOSE DIETS

I. THE INFLUENCE OF RELATIVE HUMIDITY AND DIET¹

MANUEL SCHREIBER, RICHARD ANDREW COLLINS, HIPÓLITO NIÑO-HERRERA² AND CONRAD ARNOLD ELVEHJEM

Department of Biochemistry, University of Wisconsin, Madison

TWO FIGURES

(Received for publication April 23, 1952)

INTRODUCTION

In the course of studies concerning the nutritive value of goat's milk, cow's milk and purified lactose diets, a dry scaly dermatosis was observed on the paws of young albino rats (Collins, '50; Collins, Schreiber and Elvehjem, '51). The maximum degree of dermatosis appeared when weanling rats were fed the lactose rations for about 10 to 12 days, after which time the animals spontaneously recovered in an additional two or three weeks. Although weanling rats consistently developed dermatosis, young adult animals were observed to be more resistant to scale formation when placed on lactose diets.

A mild scaly paw condition has been reported by Geyer et al. ('43) to occur in rats fed a corn oil-lactose diet "when the humidity was not abnormally high." Further studies by Boutwell et al. ('44, '45) using this corn oil-lactose diet

¹Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by funds granted by the National Dairy Council, Chicago, on behalf of the American Dairy Association and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

²Rockefeller Foundation Fellow. (From National University of Colombia, Bogota.)

confirmed the earlier observation that this diet produced scaly dermatosis. This scale formation is apparently identical to the recently observed dermatosis resulting from a cow's or goat's milk diet.

Although the addition of folic acid and vitamin B₁₂ to a mineralized goat's milk diet increased the growth rate of rats (Collins, Dietrich and Elvehjem, '50), these vitamins in addition to all other known water-soluble and fat-soluble vitamins plus ethyl linoleate gave no protection against the dermatosis (Collins, Schreiber and Elvehjem, '51).

The administration of fresh beef liver to weanling rats fed the vitamin B₁₂-low mineralized goat's milk diet resulted in an increased growth rate in accord with the vitamin B₁₂ content of this supplement, and in addition provided definite protection against the dermatosis (Collins, '50). A similar anti-dermatosis activity of beef liver was observed in rats fed mineralized cow's milk or purified lactose diets (Collins, Schreiber and Elvehjem, '51; Schreiber, '51).

In view of these preliminary observations, experiments were conducted to study various aspects of this skin problem in greater detail.

EXPERIMENTAL AND RESULTS

Male weanling albino rats 35 to 45 gm of the Sprague-Dawley strain were reared individually in raised screen-bottom cages in the air-conditioned animal room of our laboratory and in chambers at controlled humidities.

The diets were fed ad libitum and in most cases either mineralized raw cow's milk or a purified diet similar to that shown in table 1. The cow's milk was obtained from the University of Wisconsin Creamery and was fed each morning and evening to minimize souring. The milk diets were mineralized to supply 3.0 mg of iron, 0.15 mg of copper and 0.15 mg of manganese per 100 ml of milk using iron pyrophosphate, copper sulfate and manganese sulfate, respectively. All supplements were fed each morning in individual

cups, while all incorporated materials were mixed uniformly in the ration.

In all of the diets which did not contain 28% fat, adjustments were made so that the protein, vitamin and mineral constituents would be fed at the same level per calorie as the diet containing 28% fat.

TABLE 1
Composition of the purified diet

BASAL MIXTURE		WATER-SOLUBLE VITAMINS PER 100 GM OF DIET	
	%		mg
Fat	28	Thiamine	0.4
Carbohydrate	44	Riboflavin	0.6
Casein (alc.-extracted)	23.5	Pyridoxine	0.6
Salts IV ¹	4	Pantothenate (Ca)	3.0
DL-methionine	0.25	Choline	150.0
L-cystine	0.25	Niacin	1.0
FAT-SOLUBLE VITAMINS PER 100 GM OF DIET		Biotin	0.02
	mg	Inositol	40.0
A (β -Carotene)	0.56	<i>p</i> -Aminobenzoic acid	10.0
D (Calciferol)	0.014	Folic acid (Folvite)	0.50
E (α -Tocopherol)	2.24	B ₁₂ (Cobione)	0.005
K (Menadione)	0.21		

¹ Hegsted et al. ('41).

The animals were always free from dermatosis when they were received from the animal farm. They were observed daily for skin irregularities and weighed weekly. To facilitate recording the extent of dermatosis, a numerical rating was arbitrarily established.

Numerical rating	Degree of dry scaly dermatosis
0	None (normal)
1	Slight
2	Moderate
3	Severe

A series of 4 pictures (fig. 1) taken of the dorsal side of the hind paws of rats illustrates the various degrees of dry scaly dermatosis.

The following aspects of this problem have been studied: (1) seasonal variation in the degree of scaly dermatosis and its relationship to relative humidity, (2) the effect of diet composition on dermatosis, and (3) the prevention of dermatosis by beef liver and other substances.

Seasonal variation in the degree of scaly dermatosis and its relation to relative humidity

The decrease in the degree of scaly dermatosis in rats fed cow's milk during the summer of 1950 and 1951 (fig. 2) has been shown to be due to the higher relative humidity in our

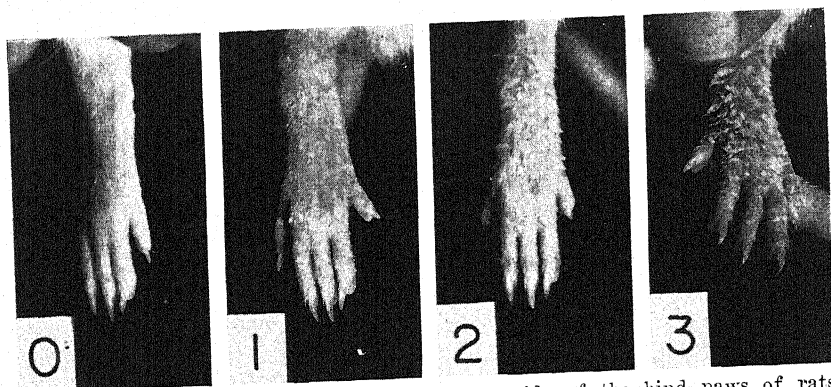


Fig. 1 A series of 4 pictures of the dorsal side of the hind paws of rats illustrating the various degrees of dry scaly dermatosis: 0 = None (normal); 1 = Slight; 2 = Moderate; and 3 = Severe.

animal room during this season. Experiments conducted at controlled low relative humidities in the chambers during the summer of 1951 demonstrated that summer milk produced a dermatosis similar to that of winter milk when the animals were maintained at comparable relative humidities. Our air-conditioned animal room is normally maintained at a relative humidity of about 30% during the winter and at 50 to 60% during the summer. The temperature is kept at approximately 26°C. throughout the year.

Low relative humidities were maintained in the chambers by controlling the inflow of dry air and by absorbing urinary

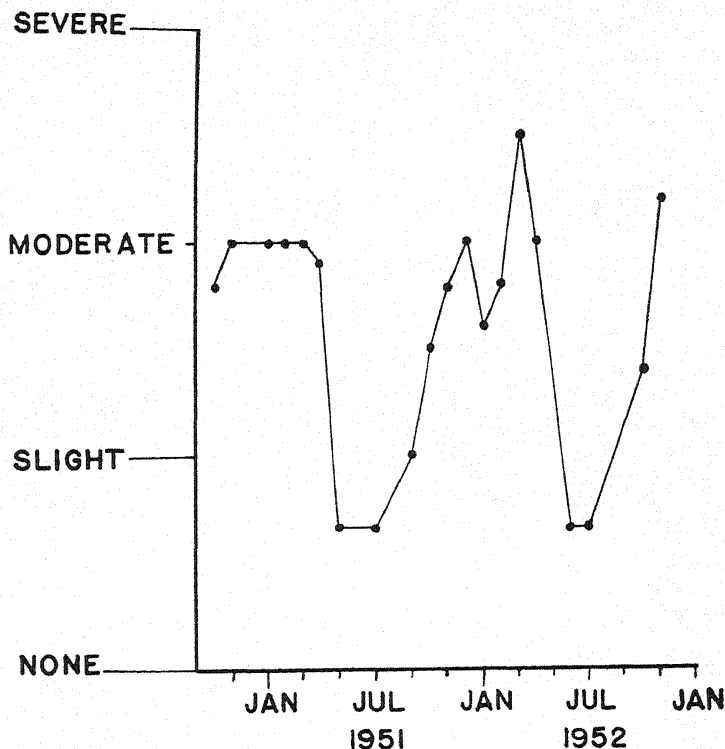


Fig. 2 The observed seasonal variation in the degree of dry scaly dermatosis in weanling rats fed a mineralized cow's milk diet.

and fecal water with anhydrous calcium chloride. The dry air was prepared by bubbling compressed air through a series of bottles containing concentrated sulfuric acid. The air was then passed through glass tubes filled with cotton and soda lime to remove acid vapors.

The minimum relative humidity which prevented the formation of the scaly dermatosis on the paws of animals fed lactose diets was a value between 50 and 60%.³ As the relative humidity was decreased, the severity of scaliness increased from zero to three as shown in figure 1.

³ Studies by Niño-Herrera, Schreiber, Collins and Elvehjem ('52) have shown that although scaliness does not occur at relative humidities above 50%, there is nevertheless histological evidence of skin irregularities.

A high humidity also accelerated recovery from the scaly dermatosis which had developed in milk-fed rats previously maintained at low relative humidity.

The effect of diet composition on dermatosis

The effect of the diet composition upon dermatosis was studied at a relative humidity of approximately 30% and at a temperature of 26°C., both in our animal room and in the humidity chambers under controlled conditions (see table 2). Rats fed cow's milk, goat's milk, corn oil-lactose, and coconut oil-lactose diets displayed a moderate degree of scaly dermatosis (groups one to 4). When butterfat-lactose or lard-lactose diets were fed only slight dermatosis was observed (groups 5 and 6). The substitution of an equimolar mixture of galactose and glucose for lactose in the corn oil diet (group 7) prevented the occurrence of dermatosis and accelerated the growth of the rats. Also, sucrose diets containing butterfat or corn oil (groups 8 and 9) did not precipitate any dermatosis. The more conventional low-fat diet containing 5% corn oil and 72% sucrose (group 10) similarly produced no dermatosis.

Changes in the level of corn oil from 20–28–35%, with corresponding reductions in lactose from 55–44–35%, had little effect in alleviating the moderate degree of dermatosis; however, the growth of the rats increased as the lactose level of the diet was decreased.

In accordance with the observations of Boutwell et al. ('45), the ceca of rats on diets containing lactose as the sole carbohydrate were larger and the contents were more fluid than the ceca from non-lactose-fed animals. Also weanling rats fed corn oil-lactose or coconut oil-lactose diets developed diarrhea which became apparent about two to 4 days after the animals were placed on experiment. Substitution of butterfat or lard for corn oil or coconut oil reduced the severity and duration of the diarrhea, whereas milk-fed animals showed little, if any, diarrhea. The presence of enlarged ceca with fluid contents and the varying degrees of diarrhea of lactose-fed

TABLE 2
The influence of diet upon dermatosis occurring on rats maintained at a relative humidity of approximately 30%

GROUP	DIET	NO. OF ANIMALS	AVE. WT. GAIN (TWO WKS.)	DERMATOSIS (TWO WKS.)	
				Incidence	Rating ²
			gms		
1	Mineralized cow's milk	265 (22) ¹	52	260/265	2.0
2	Mineralized goat's milk	38 (7)	45	38/38	2.0
3	28% corn oil—44% lactose ³	123 (17)	55	121/123	2.0
4	28% coconut oil—44% lactose	16 (3)	54	16/16	2.0
5	28% butterfat—44% lactose	59 (6)	60	48/59	1.0
6	28% lard—44% lactose	12 (2)	59	12/12	1.0
7	28% corn oil—22% galactose + 22% glucose	17 (3)	73	12/17	0.5
8	28% butterfat—44% sucrose	6 (1)	74	2/6	0.3
9	28% corn oil—44% sucrose	73 (9)	72	65/73	0.5
10	5% corn oil—72% sucrose ⁴	17 (3)	62	11/17	0.5

¹ Figures in parentheses refer to the number of experiments.

² Numerical dermatosis ratings are based on the total number of animals on each diet.

³ See table 1 for composition of this diet.

⁴ This diet is isonitrogenous on a caloric basis with respect to the 28% fat rations with similar adjustments in the vitamins and the minerals.

animals could be contributory factors in what appears to be an initial dehydration of the skin of the paws followed by the occurrence of the dry scaly dermatosis.

Other abnormalities which were frequently observed in rats fed the corn oil-lactose and coconut oil-lactose diets were: (1) blood-like material coming from the nose, and (2) thinning of the hair on the abdomen of the animal. Following the recovery from diarrhea new hair was grown on the denuded areas.

*The prevention of dermatosis by beef liver
and other substances*

As shown in table 3, the supplementation of $\frac{1}{2}$ to 1 gm of fresh beef liver/rat/day (groups 5 and 15) was found to provide excellent protection from the scaly dermatosis and also to stimulate growth; however, the diarrhea occurring in corn oil-lactose-fed rats was not alleviated. Similar results were noted when fresh beef liver was supplemented to the rats on a corn oil-lactose diet at a level of 10% of the food consumed (group 14).

The effect of humidity on the anti-dermatosis activity of liver supplements has been studied under controlled conditions (in chambers) with animals fed a milk diet. At the relative humidity range of 15-25% the protective action of liver was very slight, while at the 25-45% range liver provided definite protection against scaliness. Above 50% relative humidity the scaly dermatosis did not occur (see part I), therefore, liver could not be evaluated for its scale-preventative action under these conditions. It is known, however (Niño-Herrera et al., '52), that histological changes did occur in non-supplemented milk-fed rats at relative humidities below and above 50%.⁴

The activity of beef liver was not destroyed when aqueous homogenates were autoclaved for one hour at pH 3, 7 and 10.

Preliminary work involving the fractionation of fresh beef liver using 60% methanol provided a partially active extract

⁴ See footnote 3, page 129.

TABLE 3
Substances assayed for anti-dermatosis activity on mineralized cow's milk and 28% corn oil-44% lactose diets at approximately 30% relative humidity

BASAL DIET	GROUP NO.	MATERIAL ASSAYED	HOW ADMINISTERED	NO. OF ANIMALS	AVE. WT. GAIN (TWO WKS.)	DERMATOSIS (TWO WKS.)	
						Incidence	Rating ²
I Mineralized cow's milk	1	Non-essential amino acids ³	265 (22) ¹	gm	260/265	2.0
	2	(195 mg/day)	12 (2)	52	10/12	0.9
	3	Beef muscle shanks (1 gm/day)	Added to milk	4 (1)	61	4/4	0.7
	4	Rat liver (1 gm/day)	Supplemented	18 (1)	62	6/18	0.4
	5	Beef liver (0.5-1 gm/day)	Supplemented	69 (10)	66	35/69	0.3
II Corn oil-lactose	6	123 (17)	55	121/123	2.0
	7	Beef liver residue (5%)	Incorporated	12 (2)	63	12/12	1.8
	8	Beef liver (10%)	Incorporated	6 (1)	65	6/6	1.5
	9	Pork liver powder ⁴ (3%)	Incorporated	6 (1)	60	6/6	1.4
	10	Pork liver (1 gm/day)	Supplemented	6 (1)	65	6/6	0.7
	11	Beef kidney (1 gm/day)	Supplemented	6 (1)	59	6/6	0.5
	12	Beef muscle shanks (1 gm/day)	Supplemented	6 (1)	64	6/6	0.5
	13	Beef liver residue (5%)	Supplemented	12 (2)	63	10/12	0.5
	14	Beef liver (10%)	Supplemented	6 (1)	67	3/6	0.3
	15	Beef liver (1 gm/day)	Supplemented	59 (11)	66	31/59	0.3

¹ Figures in parentheses refer to the number of experiments.

² Numerical ratings are based on the total number of animals on each diet.

³ L-tyrosine, L-cystine, DL-serine, L-glutamic acid, DL-aspartic acid, glycine and DL-alanine were fed in a proportion roughly approximating the levels found in beef liver.

⁴ Dehydrated and defatted.

and an active residue. The residue retained its anti-dermatosis activity after ether extraction.

Liver residue and fresh liver were observed to be less active in preventing scaly dermatosis in rats when incorporated into corn oil-lactose diets at 5% and 10% levels, respectively (groups 7 and 8), than when supplemented at comparable levels (groups 13 and 14). Similar results were obtained using pork liver (see groups 9 and 10).

On a number of occasions active beef liver supplements were discontinued after two weeks of administration. The lack of storage by the rat of this anti-dermatosis activity was revealed when the moist, cool, smooth paws of liver-supplemented animals became dry, warm and rough within two to three days. This transition was followed by the development of a dry scaly dermatosis and a normal spontaneous recovery.

In addition to its role as a dermatosis-preventative agent, liver also accelerated recovery in initially non-supplemented lactose-fed animals.

Liver taken from rats which had developed the lactose-induced skin disorder possessed anti-dermatosis activity when supplemented to milk-fed animals as shown in table 3, group 4.

Those substances which were assayed by the method of supplementation to milk or corn oil-lactose diets and found to possess complete or nearly complete anti-dermatosis activity are presented in table 3. A number of other materials including animal and plant proteins were also assayed as incorporated constituents of the corn oil-lactose diet and found to have little or no protective action. However, inasmuch as the two most effective substances, fresh beef liver and liver residue, lost most of their anti-dermatosis activity when blended into the corn oil-lactose diet, all materials which were assayed by the incorporation technique must be re-assayed by the method of supplementation so that a comparable evaluation of their anti-dermatosis activity can be made with that of the beef liver supplement.

DISCUSSION

The seasonal variation in the degree of scaly dermatosis occurring in weanling rats fed milk diets (fig. 2) has been established to be the result of changes in relative humidity rather than nutritional differences in summer and winter milk. Although relative humidities above 50% have prevented the formation of visible scales, histological changes (Niño-Herrera et al., '52) were not prevented by high humidity.

As in the case of dermatoses resulting from various nutritional deficiencies, scaliness became more severe as the humidity was lowered. A situation analogous to that of the authors has been reported by Brown and Burr ('36) and Burr ('42) in which the scaly dermatosis resulting from an essential fatty acid deficiency was found to vary with relative humidity.

The purified butterfat-lactose diet produced a less severe scaly dermatosis than the natural butterfat-lactose diet (table 2, groups 5 and 1). This discrepancy may be the result of their different physical states. A dry whole milk powder diet has given variable degrees of dermatosis.

From tables 2 and 3 it is seen that the growth of animals on the non-lactose diets and the animals on liver-supplemented lactose diets was superior to that of the lactose-fed rats. From these comparisons one might surmise that a superior growth rate was instrumental in preventing the occurrence of scaly dermatosis. However, animals growing at different rates on the lactose rations exhibited the same degree of scaliness, although the recovery was more rapid among the faster growing animals. Furthermore, animals which grew at different rates on the non-lactose diets and on the liver-supplemented lactose diets did not develop dermatosis.

Beef liver supplementation to the rats fed lactose diets appeared to exert two separate effects, an anti-dermatosis action and a growth stimulation (table 3). When fresh liver or liver residue was incorporated into the corn oil-lactose

diet, the animals were not protected from the dermatosis, yet an increase in growth was noticed. Ershoff ('49) has reported a similar beneficial effect of liver upon the growth of weanling rats fed high lactose diets. The liver-supplemented rats would eat the liver within several minutes following administration, while animals receiving a diet containing incorporated liver necessarily ingested the liver at a slower rate. The rate of ingestion may, therefore, influence the anti-dermatosis action of liver.

SUMMARY

1. A dry scaly dermatosis was observed on the paws of weanling albino rats fed a mineralized milk or purified lactose diets. The maximum degree of scaliness occurred in 10 to 12 days followed by a spontaneous recovery in an additional two to three weeks.

2. The seasonal variation in the degree of dermatosis has been shown to be the result of seasonal changes in the relative humidity of our animal room rather than differences in summer and winter milks. Relative humidities above 50% prevented scaliness; decreasing values increased the severity of scaliness.

3. The purified lactose diets containing corn oil or coconut oil induced the most pronounced scaly dermatosis and diarrhea; replacement of these plant fats with butterfat or lard alleviated the dermatosis and diarrhea. Substitution of sucrose or a galactose-glucose mixture for lactose in the corn oil diet prevented the occurrence of dermatosis and diarrhea.

4. The supplementation of $\frac{1}{2}$ to 1 gm of fresh beef liver per rat per day provided definite protection against the lactose-induced (milk-induced) dry scaly dermatosis when administered within a relative humidity range of 25 to 45%. Liver was more effective as an anti-dermatosis agent when supplemented than when incorporated in the purified lactose diets.

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THE EFFECT OF PEPTONE AND AMINO ACID INGESTION UPON THE CONCENTRATIONS OF TISSUE AMINO ACIDS¹

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TWO FIGURES

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Mammalian tissues have been shown to contain significant quantities of free amino acids (Solomon et al., '51; Schurr et al., '50; Wiss and Kreuger, '49) involved presumably in the syntheses and breakdown of protein as well as in innumerable metabolic reactions. Wiss and Kreuger (Wiss and Kreuger, '49; Kreuger and Wiss, '49; Wiss, '49) recently studied the effects of high protein, high fat, or high carbohydrate diets upon individual free amino acids of liver, and Elvehjem and co-workers (Thompson et al., '50; Williams et al., '50; Denton et al., '50) measured individual tissue free amino acids following fasting, nitrogen deprivation, chilling, exercise, and amino acid deficiency. It is the purpose of the present paper to report the immediate changes in free and polypeptide amino acid concentrations in rat tissues following the ingestion of peptone. In addition, the tissues and blood plasma of rats were analyzed for individual free amino acids after the animals had been maintained upon diets supplemented with individual amino acids.

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The results indicate that the total concentrations of free amino acids in the various tissues, as well as the concentrations of the individual amino acids within a single tissue, undergo independent changes in response to the ingestion of protein or amino acids.

METHODS

Ten individual amino acids were measured by microbiological procedures previously described (Hier and Bergeim, '45; Sheffner et al., '48) except that acid produced by the growth of the organisms was measured by means of a quinhydrone electrode and the Cannon titration apparatus. The tissues were prepared for the measurement of free and polypeptide amino acids³ by the method of Solomon et al. ('51). Briefly, this consisted of immediately freezing the excised tissues with solid CO₂, pulverizing them in a mortar, and extracting the powder with boiling dilute acetic acid in a Waring Blendor. The free amino acids in the tissue extracts and in plasma were determined by analysis of their tungstic acid filtrates.

EXPERIMENTAL

*The immediate effects of peptone ingestion upon free
and polypeptide bound amino acid
levels of tissues*

Adult male Sprague-Dawley rats weighing 250 to 400 gm were maintained on a Purina Fox Chow diet and fasted 24 hours prior to the study. Two independent experiments were performed. Four rats served as controls in each experiment. The remaining rats were fed 10 gm of Difco Bactopeptone per kilogram body weight by stomach tube. The rats were successively sacrificed in pairs one-half, one, one and one-half, two, 4 and 6 hours after being fed, and their tissues analyzed for free and non-protein bound amino acids.

³ Polypeptide amino acids refer to the amino acids which become microbiologically available following hydrolysis of the tungstic acid filtrate and does not include the "free" form of the amino acids which is microbiologically available before hydrolysis.

Since the data from the two experiments were similar the results were averaged. The changes in concentration of free amino acids within the 4 tissues are presented in figures 1 and 2. In muscle, the values for arginine and lysine, which were present in high concentration in the peptone, increased greatly and those for the other amino acids showed no significant changes. The changes in levels in liver were for the

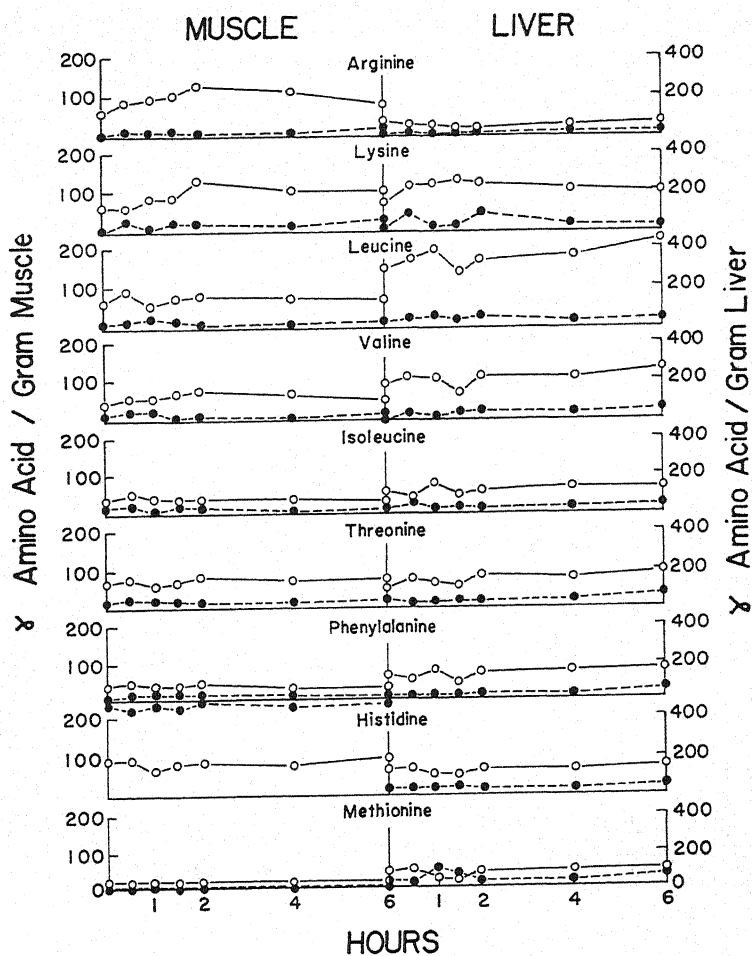


Fig. 1 Variations in the free (—) and polypeptide (---) amino acid concentrations of rat muscle and liver following the ingestion of 10 gm of peptone per kilogram body weight.

most part small, only the concentration of lysine increasing significantly; the values for lysine remained high for a prolonged period. In kidney, the free amino acid levels, other than that of leucine, rose in proportion to the quantities of each amino acid administered. Leucine increased more than the others in proportion to the amounts ingested. In general,

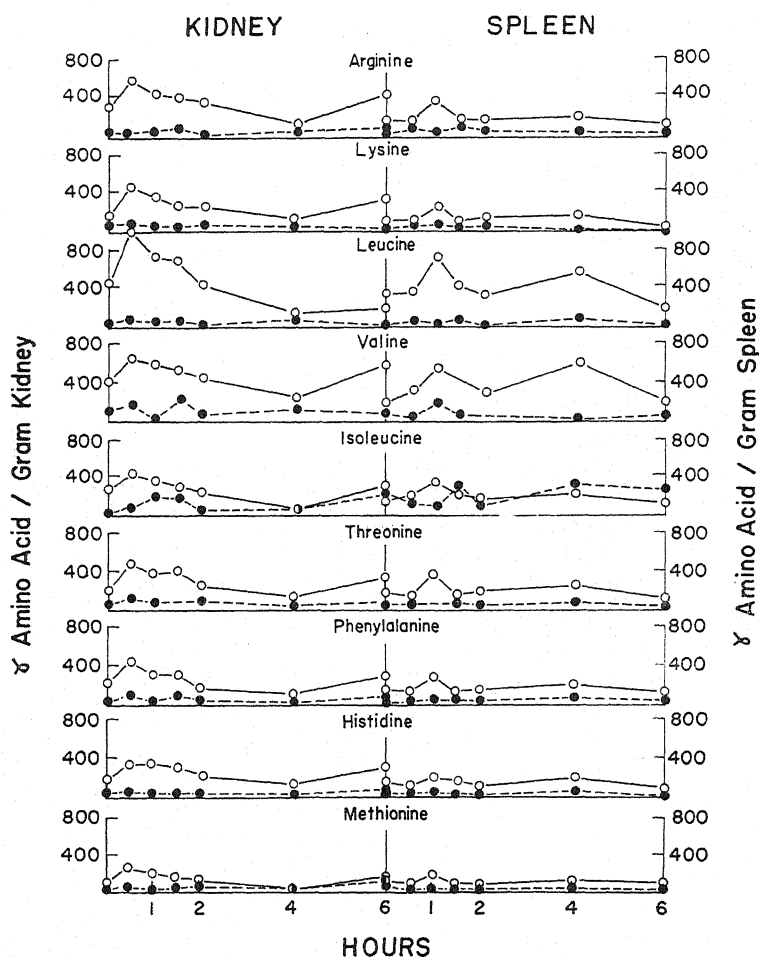


Fig. 2 Variations in the free (—) and polypeptide (---) amino acid concentrations of rat kidney and spleen following the ingestion of 10 gm of peptone per kilogram body weight.

kidney amino acid values rose in the first half hour and then fell gradually to below control values at approximately the 4th hour. There was also an immediate rise in spleen which tended to be related to the quantities ingested. The tissue amino acid levels for the two groups agreed within $\pm 15\%$.

The data obtained for the non-protein bound amino acids are also presented in figures 1 and 2. In general, values were low as compared to those for the free amino acids. The variations which resulted as a consequence of the ingestion of protein were also of a minor nature and were not significant.

*Effect of supplementing the diet with methionine
or phenylalanine*

Adult white Sprague-Dawley rats were divided into two groups of 4 each. The first group received a basal diet while the second group received, in addition, a supplement of DL-methionine which increased the methionine content of the diet from 0.3% to 3%. The basal diet had the following composition: vitamin-free casein 10%, dextrose 25%, corn starch 50.2%, peanut oil 11%, salt mixture⁴ 3%, L-cystine 0.3%; the vitamins included per kilogram were vitamin A 10,000 units, vitamin D 1,000 units, alpha tocopherol 200 mg, menadi-one 0.3 mg, riboflavin 8 mg, thiamine 8 mg, calcium pantothenate 16 mg, pyridoxine 8 mg, niacin 25 mg, biotin 0.3 mg, p-aminobenzoic acid 300 mg, inositol 2,000 mg, choline hydrochloride 2,000 mg, and folic acid 3 mg. In order to avoid unequal protein and caloric intake, the animals were fed individually at a level which coincided with the maximum intake of the animals receiving the methionine-supplemented diet. After 14 days on the described diets the rats were fasted overnight, sacrificed, and their kidney, liver, and muscle tissue and blood plasma analyzed for three amino acids, arginine, phenylalanine, and methionine. The results are pre-

⁴ Hubbell, Mendell, and Wakeman salt mixture ('37).

sented in table 1. Significant ⁵ decreases in these amino acids occurred in kidney tissue. In liver, arginine rose, phenylalanine did not change appreciably, and methionine declined in concentration. In muscle and plasma, methionine increased considerably, while arginine and phenylalanine remained essentially the same.

TABLE 1
The effect of supplementary DL-methionine in the diet upon the amino acid levels of rat tissues and plasma

AMINO ACID	KIDNEY		LIVER	
	Control	Supplemented	Control	Supplemented
	$\mu\text{g/gm}$		$\mu\text{g/gm}$	
Arginine	153 ± 9.1^1	76 ± 7.7	19 ± 1.0	29 ± 2.2
Phenylalanine	116 ± 6.9	75 ± 5.4	71 ± 3.2	67 ± 3.9
Methionine	4.1 ± 0.9	0.8 ± 0.3	4.5 ± 0.7	1.3 ± 0.3
AMINO ACID	MUSCLE		PLASMA	
	Control	Supplemented	Control	Supplemented
	$\mu\text{g/gm}$		$\mu\text{g/ml}$	
Arginine	66 ± 5.1	57 ± 5.3	24 ± 2.1	20 ± 3.0
Phenylalanine	14 ± 1.5	15 ± 1.4	7.1 ± 0.5	8 ± 0.5
Methionine	0.5 ± 0.2	9.3 ± 3.5	3.2 ± 0.1	9.2 ± 1.5

¹ Standard error of the mean.

A second series of experiments was performed similar except that the basal diet contained 15% casein and the experimental diet contained in addition DL-phenylalanine to increase the phenylalanine content of the diet from 0.77% to 3.85%. The results of these experiments are presented in table 2. Contrary to what occurred on the high-methionine diet, on the phenylalanine-supplemented diet the fasting kidney arginine and phenylalanine levels rose significantly. The rise in kidney methionine was not statistically significant.

⁵ Tests of significance were calculated from the formulae (Dixon and Massey, '51):

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S_p \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}; \quad S_p^2 = \frac{N_1 S_1^2 + N_2 S_2^2}{N_1 + N_2 - 2}$$

Also, in contrast to what occurred on the methionine supplemented diet, liver arginine decreased appreciably. In muscle tissue, decreases in arginine and methionine occurred on the high phenylalanine diet, but no changes were noted in the phenylalanine concentration. The amino acid levels in plasma remained essentially constant.

TABLE 2
The effect of supplementary DL-phenylalanine in the diet upon the amino acid levels of rat tissues and plasma¹

AMINO ACID	KIDNEY		LIVER	
	Control	Supplemented	Control	Supplemented
	$\mu\text{g/gm}$		$\mu\text{g/ml}$	
Arginine	103 ± 5.8^2	130 ± 8.7	34 ± 2.3	24 ± 1.4
Phenylalanine	74 ± 2.9	113 ± 7.7	54 ± 2.7	48 ± 2.5
Methionine	1.8 ± 0.5	2.9 ± 0.6	7.8 ± 0.7	6.2 ± 0.7

AMINO ACID	MUSCLE		PLASMA	
	Control	Supplemented	Control	Supplemented
	$\mu\text{g/gm}$		$\mu\text{g/gm}$	
Arginine	67 ± 2.0	52 ± 2.9	27 ± 1.0	27 ± 1.4
Phenylalanine	13 ± 0.6	13 ± 0.7	8.3 ± 0.6	8.0 ± 0.6
Methionine	5.9 ± 0.4	3.2 ± 0.3	5.6 ± 0.5	5.2 ± 0.3

¹ Each mean value represents the analyses from 4 rats.

² Standard error of the mean.

DISCUSSION

The response of the individual free amino acid levels in muscle to the ingestion of peptone corresponded to the quantity of the respective amino acids ingested. In liver, the changes were for the most part small. The prolonged high level of lysine probably reflects the slower rate at which lysine is metabolized in liver, whereas the decrease in arginine values indicate the increased arginase activity which occurs postabsorptively and which is associated with urea formation. In kidney, the free amino acid levels, other than for leucine, rose in proportion to the quantities of each amino acid administered; leucine increased more than the others in proportion to the amounts ingested. The relatively high levels of leucine in all the tissues after the ingestion of food

indicated that this amino acid is not utilized as rapidly as the others studied; in particular, the prolonged high concentrations in liver suggest that it is not destroyed as rapidly as the other amino acids.

An interesting observation made during this study was the fact that the large postprandial variations in free amino acids in kidney and spleen were accompanied by similar changes in the quantity of non-heat coagulable water soluble proteins which could be extracted from these tissues at pH 6. It could not be ascertained from the data whether or not the occurrence of larger quantities of these proteins was due to increased synthesis during the postprandial period of high-free amino acid levels.

The administration of a diet containing a high concentration of methionine resulted in decreased fasting kidney amino acid levels, including methionine. It is possible that amino acid catabolyzing enzymes were stimulated or increased in quantity. Data presented elsewhere (Sheffner and Bergeim, to be published) indicated that kidney L-amino acid oxidase activity is increased on a high methionine diet. However, since kidney L-amino acid oxidase is relatively low, it is likely that a general stimulation of catabolizing enzymes was responsible for the observed decrease in amino acid levels.

In the course of this work it was also noted that the free amino acid levels of leucine, valine, and threonine were significantly higher in muscle tissue of rats maintained upon a whole milk and bread diet than in the muscle of rats fed a Purina Chow diet containing considerably more of these amino acids.

The results therefore indicate that the concentration of free amino acids in tissues frequently undergoes changes in response to the ingestion of a protein, or amino acids which are unrelated to the amount of the particular amino acid ingested, and that single amino acids or various combinations of amino acids in the diet can influence the rate at which amino acids are utilized by the tissues.

SUMMARY

1. At various intervals of time following the ingestion of relatively large quantities of peptone, determinations were made in rats of the concentrations of 9 individual free amino acids in femoral muscle, liver, kidney, and spleen. In addition, measurements were made of variations in the individual non-protein bound amino acids of these tissues. The results indicate that the concentrations of individual amino acids within a single tissue undergo independent changes in response to the ingestion of peptone.

2. Supplementation of normal diets with large quantities of DL-methionine resulted in increased concentrations of methionine in plasma and muscle, but lower levels in kidney and liver tissue. Arginine and phenylalanine levels also decreased in kidney. On a diet containing high concentrations of DL-phenylalanine, no significant changes occurred in muscle and liver tissue and in plasma; contrary to what occurred on the high methionine diet, the levels of arginine and phenylalanine rose in the kidneys of fasted rats; kidney methionine concentrations did not change appreciably.

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THE EFFECT OF HEAT ON THE LYSINE AND METHIONINE IN SUNFLOWER SEED OIL MEAL^{1, 2}

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The principal deficiency in commercial sunflower seed oil meal when used as a supplementary protein source in practical type diets for chicks and poults appears to be lysine (McGinnis, Hsu and Carver, '48; Slinger, Hill, Gartley and Branion, '49). The latter workers used an expeller-processed sunflower seed oil meal which analyzed 2.7% lysine on a 16% nitrogen basis. This value may be compared with 3.8% found by Block and Bolling ('45) in a solvent-processed meal. The evident discrepancy could be attributed to lysine destruction caused by exposure of the meal to high temperatures during the expeller processing, and is not unexpected in view of the conclusion of Clandinin and Robblee ('50) that the great variability in the nutritive value of commercial sunflower seed meals results from excessive processing temperatures.

The experiments described, herein, were conducted to study the effects of controlled conditions of heating on the lysine and methionine in sunflower seed oil meal. There is no evidence in the literature to suggest that methionine is deficient

¹ The data reported in this paper are taken in part from a thesis presented by J. C. Alexander in partial fulfillment of the requirements for the degree of Master of Science in Agriculture, University of Toronto, 1951. The investigation was supported in part by a grant from Toronto Elevators Ltd., Toronto, Ontario, and J. C. Alexander was holder of a fellowship from the Ontario Research Council.

² The authors are indebted to Co-op Vegetable Oils Ltd., Altona, Manitoba, for supplying the sunflower seed materials.

³ Present address: Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

in commercial sunflower seed oil meal from a nutritional standpoint. However, studies on this amino acid were included in view of the many published reports dealing with the effects of heat on its availability in other proteins.

MICROBIOLOGICAL STUDIES

Experimental

Cleaned dehulled advance Hybrid sunflower seeds⁴ were coarsely ground, extracted for 24 hours in a soxhlet apparatus with diethyl ether and then finely ground. In one series of experiments the meal was heat-treated after extraction of the oil, and in a second series it was heat-treated before extraction.

Following the heat treatment the material was subjected to both acid and enzymatic hydrolysis. Acid hydrolysis was accomplished by autoclaving at 15 lb. pressure for 8 hours with 10% hydrochloric acid. Enzymatic hydrolysis was accomplished with pancreatin, by a procedure similar to that described by Melnick and Oser ('49) except that a borate buffer was used rather than a phosphate buffer. The enzymatic digestions were carried out for 120 hours at which time essentially maximum release of amino nitrogen and methionine had taken place.

Microbiological assays for lysine and methionine were conducted on the acid hydrolysates and for methionine on the enzymatic hydrolysates. *Leuconostoc mesenteroides* P-60 was employed for lysine and *Streptococcus faecalis* no. 9790 for methionine. The medium and procedure was that of Henderson and Snell ('48) with some modifications. Tween 80⁵ was added to the medium at levels of 0.02% to 0.1% and assays were conducted in a total volume of 4 ml per assay tube. Amino nitrogen was measured as described by Melnick and Oser ('49).

⁴Previous to dehulling, the seeds had been passed through a Morris drier which dried them to a moisture content of about 7%.

⁵Polyoxyethylene sorbitan monooleate. Purchased from the Atlas Powder Co. Ltd., Brantford, Ontario.

When heat treatments were applied following extraction, the samples were heated in the containers used for the hydrolysis and these heated portions then hydrolyzed in toto. For acid hydrolysis 0.1 gm lots in pyrex test tubes were used, and for enzymatic hydrolysis, 6 gm in 125 ml erlenmeyer flasks.

When heat treatments were applied before extraction, the ground material was heated on metal trays in layers not exceeding one-half inch. There was a small variation in the protein content of the heated samples in this series. Accordingly, for comparative purposes all analytical values reported are based on the protein content ($N \times 6.25$) of the unheated meal.

Results and Discussion

The data in table 1 show that autoclaving and dry heating caused considerable destruction of lysine. The amount of destruction is roughly proportional to the duration of heating. It is also apparent that the presence of the oil in the meal during the heat treatment afforded some protection against lysine destruction during autoclaving for 15 and 30 minutes and during dry heating. The amount of destruction of methionine is negligible for all treatments. Some destruction is evident during heating before extraction of the oil, but even in this case the destruction found did not exceed 10%.

The susceptibility of lysine to moist heat destruction and the relative stability of methionine is in accord with similar investigations on soybean oil meal by Riesen, Clandinin, Elvehjem and Cravens ('47); Clandinin, Cravens, Elvehjem and Halpin ('47) and Evans and McGinnis ('48). However, it has been shown by Evans and Butts ('48) that dry heating of soybean oil meal is considerably less destructive to the lysine in the meal than is moist heating. In this respect, at least, soybean oil meal and sunflower seed oil meal respond differently to heat treatment.

Table 2 summarizes the findings on the release of methionine from sunflower seeds by enzymatic digestion. The striking thing about the data in table 2 is that for every sample

the percentage of the total methionine released exceeded the percentage hydrolysis of the protein. This would suggest that the methionine is being released in a form which is more active for streptococcus faecalis than is the free amino acid.

TABLE 1

Effect of heat on the destruction of lysine and methionine in sunflower seeds

HEAT TREATMENT ¹	TREATED AFTER ETHER EXTRACTION				TREATED BEFORE ETHER EXTRACTION			
	Lysine		Methionine		Lysine		Methionine	
	Con- tent	De- stroyed ²	Con- tent	De- stroyed ²	Con- tent	De- stroyed ²	Con- tent	De- stroyed ²
	%	%	%	%	%	%	%	%
None	1.80		0.96		1.80		0.96	
Autoclaving 15 minutes	1.56	13	0.96	0	1.73	4	0.89	7
Autoclaving 30 minutes	1.48	18	0.97	0	1.80	0	0.90	6
Autoclaving 2.5 hours	1.51	16	1.00	0	1.48	18	0.87	9
Autoclaving 5 hours	1.25	30	0.96	0	1.13	37	0.87	9
Autoclaving 10 hours	0.66	63	0.92	4	0.84	53	0.93	3
Dry heating 2.5 hours	1.38	23	0.93	3	1.72	4	0.93	3
Dry heating 5 hours	1.07	40	0.95	1	1.30	28	0.90	6
Dry heating 10 hours	0.74	59	0.92	4	1.17	35	0.93	3

¹ Autoclaving at 15 lb. pressure and dry heating in a hot air oven at 121°C.

² Decreases in the lysine and methionine content are considered to represent destruction of the amino acids.

Pader, Melnick and Oser ('48) have found evidence that peptides of lysine may produce a growth stimulation of assay organisms greater than that produced by an equivalent quantity of free lysine. On the other hand, methionine may be released quite early during enzyme digestion as compared

with the other amino acids. However, in experiments not reported here the authors have found a similar lack of agreement for lysine. This would suggest rather strong limitations on the use of microbiological assays for examining the amino acid content of incompletely hydrolyzed enzymatic digests.

Subject to these limitations, however, there is no clear evidence of extensive alteration of the methionine by heat treatment into a form from which biologically active methionine was not liberated by *in vitro* enzyme hydrolysis. Only

TABLE 2
Release of methionine from sunflower seeds by enzymatic hydrolysis

HEAT TREATMENT	TREATED AFTER ETHER EXTRACTION			TREATED BEFORE ETHER EXTRACTION		
	Per cent hydroly- sis ¹	Per cent methionine found ²	Per cent of total methio- nine ³	Per cent hydroly- sis ¹	Per cent methionine found ²	Per cent of total methio- nine ³
None	47	0.71	74	48	0.68	72
Autoclaving 15 minutes				29	0.69	70
Autoclaving 30 minutes				34	0.72	80
Autoclaving 2.5 hours	46	0.91	91	37	0.72	83
Autoclaving 5 hours	44	0.74	77	41	0.70	80
Autoclaving 10 hours	33	0.52	56	37	0.58	62
Dry heating 2.5 hours	40			38	0.58	62
Dry heating 5 hours	40	0.66	61	40	0.66	73
Dry heating 10 hours	37			42	0.71	76

¹ Formol titratable nitrogen as a percentage of the amount released from the unheated meal by hydrolysis with 8 N H₂SO₄ for 24 hours.

² Following 120 hours pancreatic digestion.

³ Total methionine is assumed to be that found following acid hydrolysis of a similarly treated meal.

with 10 hours of autoclaving and two and one-half hours' dry heating does it appear that such an alteration has taken place. By these treatments, the methionine released as per cent of the total has been reduced from 74 to 56% for 10 hours' autoclaving following extraction of the oil, from 74 to 62% for 10 hours' autoclaving previous to extraction, and from 74 to 62% by two and one-half hours' dry heating. With milder autoclaving treatment the quantity of methionine released was actually increased.

CHICK GROWTH STUDIES

Experimental

Three feeding experiments were conducted in which the method of experimentation was similar. Day-old chicks were fed a commercial chick starter diet for a period of either 11 days or 14 days. They were then divided into experimental groups of equal numbers in such a way that each group was of approximately the same weight. In this allocation procedure extremely large and extremely small birds were discarded. The test diets were assigned to these groups and the gain in weight for the succeeding two weeks was taken as the criterion of response. During both the preliminary and experimental feeding periods the chicks were housed in battery brooders in an air-conditioned animal laboratory.

The experimental diets used were of a practical type but proved adequate for revealing lysine deficiencies. They were compounded by supplementing a basal diet with variously treated meals. The basal diet was of the following percentage composition of the final diets: ground corn 25.5, ground oats 12.5, ground oat groats 6.5, ground barley 6, ground wheat 15, dehydrated alfalfa meal 3, iodized salt 0.60, calcium carbonate 1, tricalcium phosphate 1.5, fortified fish oil (2400 A, 400 D) 0.24, manganese sulphate tetrahydrate 0.02, choline chloride (25% concentrate) 0.19, vitamin B₁₂ supplement^a 0.005, and riboflavin 0.1 gm per 100 lb. of diet.

^a APF-3 supplied by Merck and Co. Ltd., Montreal, Quebec.

For each diet the balance to make 100% was composed of the variously treated meals, lysine when added, and a small additional amount of corn not exceeding 1.9% in any case. The meals were added in amounts to provide the comparable protein contents given in table 3.

In experiment 1, tests were conducted on a commercial sunflower seed oil meal. This experiment was conducted primarily to test the value of the experimental method to satisfactorily detect a lysine deficiency. In experiments 2 and 3 the solvent process meal that was used was prepared by a low temperature hexane extraction during which the maximum temperature was 160°F.⁷

The commercial expeller meal⁸ used in experiment 2 was prepared from the same seeds as the solvent meal.

The meals were spread in thin layers on metal trays and then heated. All diets were fed ad libitum.

Results and Discussion

Table 3 gives the results of the three experiments. Only the mean gains in weight over the two-week experimental period are given. However, marked differences in the mean gain among the groups on the various diets were evident even at the end of the first week.

It is clear that the commercial expeller-process sunflower seed oil meals used in these experiments were markedly improved by supplementation with lysine (experiment 1, diets 1 and 2; experiment 2, diets 8 and 9). It is also evident that considerable lysine in the solvent process meal was destroyed or otherwise rendered unavailable to the chick by one-hour and two-hour autoclaving (experiment 2, diets 1, 2, 4, 5, 6 and 7). The solvent-process meal autoclaved one hour with added lysine promoted even better growth than the unheated meal. This finding indicates that one-hour autoclaving did

⁷ Prepared by the research laboratories of Canadian Breweries Ltd., Toronto, Ontario, through the courtesy of Dr. W. E. Parker.

⁸ Prepared by Co-op Vegetable Oils Ltd., Altona, Manitoba.

TABLE 3

Effect of heating on the feeding value of sunflower seed oil meal for chicks

EXPERI- MENT ¹ NO.	DIET SUPPLEMENT TO BASAL DIET	PROTEIN IN DIET (N × 6.25)	LYSINE ² IN DIET	MEAN GAIN ³ FOR TWO WEEKS	FEED- GAIN RATIO
		%	%	gm	
1 ⁴	1 Commercial meal (expeller process)	23.9	0.5	75	5.34
	2 As 1 plus 0.4% L-lysine HCl	23.2	0.83	108	3.93
	3 As 1 autoclaved 1 hour at 15 lb.	23.5	0.45	47	7.94
	4 As 3 plus 0.4% L-lysine HCl	23.8	0.78	120	3.13
2	1 Solvent process meal	20.3	0.69	132	2.64
	2 As 1 plus 0.4% L-lysine HCl	20.2	1.02	141	2.46
	3 As 1 heated 30 min. at 100°C.	19.7	0.66	129	3.17
	4 As 1 autoclaved 1 hour at 15 lb.	19.8	0.55	80	4.48
	5 As 4 plus 0.4% L-lysine HCl	20.2	0.88	149	2.50
	6 As 1 autoclaved 2 hours at 15 lb.	19.6	0.37	51	5.87
	7 As 6 plus 0.4% L-lysine HCl	20.5	0.70	129	2.56
	8 Commercial meal ⁴ (expeller process)	19.5	0.55	79	4.02
	9 As 8 plus 0.4% L-lysine HCl	20.3	0.88	147	2.63
	10 Commercial soybean oil meal (solvent)	20.3	0.91	149	3.07
3	1 Solvent process meal	19.7		109	2.6
	2 As 1 autoclaved 5 min.	19.4		101	4.27
	3 As 1 autoclaved 10 min.	19.2		99	3.15
	4 As 1 autoclaved 20 min.	20.6		102	3.05

¹ Each group in experiment 1 contained 17 ♀ chicks, in experiment 2, 18 ♀ chicks, and in experiment 3, 8 ♂ chicks and 8 ♀ chicks. Chicks in experiments 1 and 2 were New Hampshire Barred Rock cross and in experiment 3, White Leghorns.

² For diets with added lysine, the lysine contents shown are the sum of the amount found by analysis of the unsupplemented diet plus the added lysine.

³ Eleventh to the 25th day in experiments 1 and 2 and 14th to 28th day in experiment 3.

⁴ Basal contained 0.2% added DL-methionine.

not affect nutrients in the meal other than lysine to any practical degree for the type of diet used.

There is evidence from the data that rather severe heating of the meal may actually improve its nutritional value provided the resulting lysine destruction has been compensated for (experiment 1, diets 2 and 4; experiment 2, diets 5 and 9). This improvement is small but consistent in the two experiments. Since the diets in experiment 1 contained added methionine, it seems unlikely that this result could be attributed to an increase in the availability of methionine.

In view of the well established fact that mild heat treatment improves the nutritional value of soybean oil meal, several relatively mild heat treatments were applied to the solvent process sunflower seed oil meal. Specifically, Evans, McGinnis and St. John ('47) showed that heating soybean oil meal for 30 minutes at 100°C. improved its nutritional value. Similar treatment in experiment 2 (diets 1 and 3) and autoclaving for 5, 10 and 20 minutes in experiment 3 failed to reveal any increase in growth promoting value.

It is of interest to note that no feather abnormalities in shape or color were observed on any of the lysine-deficient diets. Fritz, Hooper, Halpin and Moore ('46), Slinger, Hill, Gartley and Branion ('49), and Gartley, Slinger and Hill ('50) have reported that a lysine deficiency in the diet of Broad-Breasted Bronze poults results in poor pigmented feathers. The cross used in experiments 1 and 2 has dark colored feathers, which would readily have revealed any repigmentation.

Microbiological assays of the various sunflower seed oil meals used in feeding experiments 2 and 3 showed that autoclaving the solvent-process meal 30 minutes at 100°C., one hour at 15 lb. pressure, and two hours at 15 lb. pressure destroyed 10%, 21% and 51% of the lysine, respectively, and that the commercial expeller process meal had 30% less lysine than the solvent-process meal. The heat destruction of lysine thus recorded is considerably greater than that found with the meal used in the microbiological studies. Table 1 shows

30% destruction after autoclaving 5 hours at 15 lb. pressure. The sunflower seeds used in these two studies were from different crops and also the procedure used in preparing the extracted meals differed in the two cases. It may also be of significance that the sunflower seed material used in feeding experiments 2 and 3 was taken from a crop which was late in maturing and therefore contained many immature seeds. In any event the results suggest that there are factors which markedly influence the amount of destruction which may result from a given heat treatment.

SUMMARY

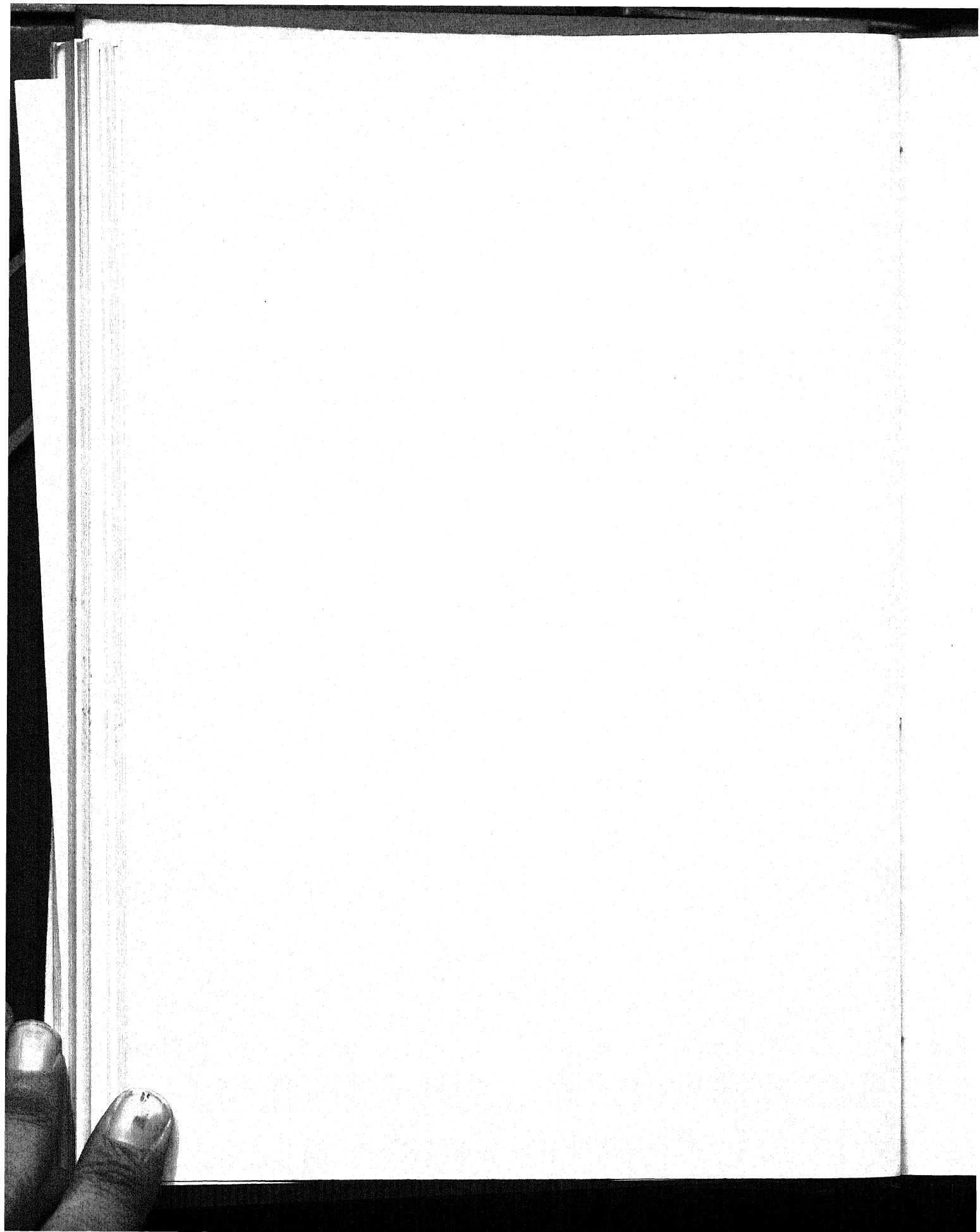
Autoclaving at 15 lb. pressure and dry heating at 121°C. destroyed a large proportion of the lysine in sunflower seed oil meal. Neither type of heating had an appreciable effect on methionine.

The findings support the conclusion that destruction of lysine during processing is probably the chief cause of the poor nutritional value for chick diets of commercial expeller-process sunflower seed oil meal.

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THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

IX. THE RELATIONSHIP OF RADIATION INJURY IN THE RAT TO THE FAT CONTENT OF THE DIET^{1,2}

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TWO FIGURES

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Within the last few years, evidence has accumulated demonstrating the importance of fat in the diet (Deuel and Greenberg, '50). Rats receiving a generous quantity of fat in the diet were more resistant to such forms of stress as exercise (Deuel et al., '47; Samuels et al., '48), fasting (Samuels et al., '48), or hyperthyroidism (Greenberg and Deuel, '50) than were animals on a fat-low regimen. The present studies were undertaken to determine whether susceptibility to another type of stress, namely irradiation injury, might also be influenced by diet.

A number of investigations have shown an increase in susceptibility to x-ray injury when deficient diets are administered. Thus, Johnson et al. ('46) reported that young rats exhibited a markedly increased susceptibility to irradiation injury when fed a vitamin-free diet over that exhibited when the animals were maintained on an adequate dietary regimen.

¹ This work was carried out under a contract between the University of Southern California and the Atomic Energy Commission (No. AT(11-1)-113).

² Contribution 310 of the Department of Biochemistry and Nutrition, University of Southern California.

A considerable degree of protection could be obtained by daily oral supplementation with the B vitamins, given the rats prior to the x-irradiation. Thiamine has been reported to combat the nausea produced by irradiation injury in man (Wallace, '41; Whitmore, '43). In fact, Whitmore suggested that thiamine is required for the metabolism of the products of nuclear disintegration or of other substances arising as a result of irradiation.

Many substances have been used in attempts to modify irradiation sickness. Pyridoxine does not appear to influence the onset, severity, or duration of the leukopenia following irradiation, but it does afford a definite protection against delayed effects of the Roentgen rays. Goldfeder et al. ('48) found that, if the leukopoietic system of their experimental animals returned to normal, they did not die of delayed reactions. In this respect, pyridoxine hydrochloride resembled folic acid. Both these substances decrease, and in some cases prevent, diarrhea in irradiated mice. They may also extend the survival time of the animals. On the other hand, most reports on folic acid have been negative. These studies concerned principally leukopenia in cats (Adams and Lawrence, '48), swine (Cronkite et al., '50), and rats (Jacobson et al., '48; Stearner and Jacobson, '47; Stearner, '48). Carter and co-workers ('50) have likewise demonstrated that crystalline vitamin B₁₂ does not prolong the survival of rats exposed to x-rays.

Ershoff ('52) has reported that the survival time of immature and young female rats following multiple sublethal doses of x-irradiation was significantly prolonged on a liver-containing diet over that on a ration high in the known synthetic B vitamins. However, no significant differences were noted in survival time between the rats on the several dietary regimens when a single lethal dose of x-irradiation was employed.

Protein-depleted rats have been reported to be more susceptible to penetrating radiation than are animals on a diet containing adequate protein (Jennings, '50). This finding

is in line with the observation of Ross and Ely ('49) that the metabolism of tyrosine, tryptophan, and arginine is upset by x-irradiation.

Protection from x-irradiation injury has also been obtained by the use of specific compounds known to be important in the functioning of the epidermis. Thus, beneficial effects have been reported in a number of cases with rutin and other compounds resembling vitamin P and having a flavonoid structure. Griffith et al. ('47) and Sokoloff and co-workers ('50a, b) obtained positive results with rats, while Rekers and Field demonstrated increased survival in dogs subjected to low dosages of x-irradiation ('48; Field and Rekers, '49); however, the latter workers ('48) did not find that the flavonoids exerted any beneficial effects when high dosages of x-irradiation were used. Clark and collaborators ('48) have reported that a flavonoid prepared from lemon protected guinea pigs against x-irradiation damage. However, Cronkite and associates ('49, '51b) have consistently noted negative results in the case of mice treated with flavonoid compounds, as have also Kohn et al. ('48) working with rats. Haley and Mann ('52) have also recently reported negative results from the use of rutin in protecting guinea pigs from x-ray injury. There is no evidence that the positive results obtained with citrus products is to be attributed to ascorbic acid, since tests with this substance have yielded only negative results (Simmons et al., '46; Field and Rekers, '48, '49).

Cysteine and related sulfur derivatives are compounds of importance in relation to the epidermis which have likewise been shown to be active in protection from x-irradiation. Cysteine was found to be an effective protective agent against x-irradiation when given intravenously or orally to rats or mice (Patt et al., '49, '50a, b; Goldie et al., '51; Swift et al., '52). Patt, Smith and Jackson ('50a) found that intravenous injections of cysteine prior to irradiation reduced the mortality rate from 66 to 22%; they also observed that the leukopenia and erythropenia were less severe and of shorter duration in the pretreated rats. Similar results were also reported

by Smith et al. ('50). This amino acid appeared to be active when given at considerable periods prior to exposure to x-rays, but was ineffective when given shortly after irradiation. Apparently it is necessary for this substance to be in the blood and tissues at the time of exposure to the Roentgen rays in order to counteract their effects.

Some improvement in survival time, and a decrease in weight loss, in rats after x-irradiation, followed treatment with the cysteine derivative, glutathione (Chapman and Cronkite, '50; Chapman et al., '50), although the results on mice were essentially negative (Cronkite, Brecher and Chapman, '51a).

BAL has also been shown to have a protective action against x-irradiation in mice (Works et al., '50) and in rats (Hursh, '49). Another sulfur compound, thiourea, has likewise been found to possess some potency as a protective agent in preventing x-ray injury in mice (Limperos and Mosher, '50), although Haley et al., ('50) reported negative results in tests on a strain of mice which differed from that used by the former investigators. The recent reports of Selle ('49, '52), listing all the agents which have been used in an attempt to counteract the effects of ionizing radiations, also include a discussion of the effects of cyanides, nitrides, antibiotics, antihistaminics, adrenocortical extracts, sex hormones, splenic implants, and environmental factors.

Decker et al. ('50) reported typical symptoms of acute essential fatty acid deficiency produced by x-irradiation in fat-depleted mice. They also noted a greater survival of mice on a fat-free diet plus corn oil than on the control diet. The present experiments were carried out to investigate the effect of dietary fat on injury due to exposure to x-irradiation.

EXPERIMENTAL

Three series of tests were carried out on rats receiving diets varying in fat content, to determine the effect of a single large dose or of repeated sublethal doses of x-irradiation on

survival, changes in body weight, hemoglobin and white cell count.

In series I, a single dose of x-irradiation was given at levels of 650 or 850 *r* to young male and female rats (approximately 10 to 12 weeks of age) which had received a fat-free diet (diet 1) or a high-fat diet (diet 4) for three to 4 weeks prior to the tests. The higher level of irradiation was such that one would expect a mortality of 50% within 30 days after exposure to the rays. In series II, adult rats which had previously received a fat-free diet (diet 1), or diets containing large amounts of fat (diets 3 and 4), from weaning at three weeks of age for 15 months, were exposed to 300 *r* weekly for 4 weeks; later, from the 7th to the 13th weeks, they were exposed on alternate weeks.

The tests described as series III were carried out on young rats in a manner somewhat similar to that employed in series II. Upon weaning, the rats were placed on diets 1 and 4. After receiving this regimen for three weeks, they were redistributed into three groups which were fed diets 1, 2 (2% fat) and 4 for the rest of the test. After 6 weeks on the new diet, the animals (at the age of 12 weeks) were subjected to the first exposure of 300 *r*. Control groups which received no irradiation were included in series III.

The rats were of the U.S.C. strain. They were kept in individual cages and were weighed daily. Blood for hemoglobin determination and white cell count was obtained from the tail. In order to allow sufficient time for the tail to heal, and to lessen the probability of infection, samples were removed from the same rat only once in two or three weeks. Hemoglobin was determined by the use of the Haden-Hauser hemoglobinometer, and white cell counts were carried out by the usual procedure, using a Spencer Bright Line hemocytometer. The control leukocyte counts obtained before irradiation were the average figures obtained on all the rats of each group. The mean value of the controls was used for the control value in calculating the decrease in leukocyte

count after x-irradiation on the individual rats. Values for hemoglobin could not be determined below a value of 7.5%.

The composition of the experimental diets used is included in table 1. The control diet was a commercial laboratory ration,³ which contains approximately 5% fat.

TABLE 1
Composition of diets

COMPONENTS ¹	DIET			
	1	2	3	4
	%	%	%	%
Casein, commercial	25.0	25.2	29.5	34.0
Sucrose	66.84	65.64	46.31	26.28
Cottonseed oil ²	0	1.0	14.0	29.0
Cellulose ³	3.0	3.0	4.0	4.0
Salt mixture ⁴	4.0	4.0	5.0	5.5
Water-soluble vitamin mixture ⁵	0.16	0.16	0.19	0.22
Fat-soluble vitamin mixture ⁶	1.0 ⁷	1.0	1.0	1.0

¹ The folic acid was kindly furnished by Lederle Laboratories and the biotin by the Hoffmann-LaRoche Co. The remaining B vitamins and α -tocopherol were given to us through the kindness of Merek and Co. Crystalline vitamin D₂ was generously furnished us by the Winthrop Chemical Co.

² Wesson oil.

³ "Solka-floc" obtained from the Brown Company, San Francisco.

⁴ Osborne-Mendel salt mixture (*J. Biol. Chem.*, 1917, 32: 309).

⁵ The water-soluble vitamin mixture had the following composition: choline chloride, 91.5%; thiamine chloride hydrochloride, 1.24%; riboflavin, 1.24%; pyridoxine hydrochloride, 1.24%; calcium pantothenate, 2.48%; nicotinic acid, 1.83%; folic acid, 0.30%; and biotin, 0.01%. Vitamin B₁₂ (dissolved in ethyl alcohol) was added to the extent of 0.01%. This also contains 0.15% menadione.

⁶ The cottonseed oil solution of fat-soluble vitamins had the following composition per 100 gm: α -tocopherol, 500 mg; carotene (90% — β and 10% — α), 20 mg; crystalline vitamin D₂, 0.5 mg.

⁷ Made up in propylene glycol instead of cottonseed oil.

The irradiation of the rats was carried out by the use of a General Electric Maximar 250-III 200/260 volt apparatus.⁴ The radiation chamber was a wooden box, having a plastic top made of cellulose acetate sheets $\frac{1}{8}$ inch in thickness, which was used to hold the rats during exposure to the x-rays. This

³ Purina laboratory chow, Ralston Purina Co., St. Louis, Mo.

⁴ For a more complete description of this apparatus, see Ershoff ('52).

was divided into a number of compartments, with cellulose acetate sheeting, which were large enough to allow the rats to remain in them without discomfort but too small to permit any change of position. The intensity of the irradiation was standardized by measurement with a Victoreen r meter within the compartments. The plastic cover was found to absorb only 1.5% of the irradiation. The calculation of the dosage is based upon the measurement made on the top of the radiation chamber. If the measurement is made on the floor of the chamber with the cover in place, the value is approximately 18% lower.

During the period in which the rats were undergoing x-ray treatment, the radiation chamber was constantly rotated at a slow speed (2 R.P.M.) on a rotatory table. This ensured an equal distribution of x-irradiation in all positions within the box. To prevent overheating during the x-ray treatment, a fan, directed toward the box, was operated whenever irradiation was in progress. Numerous tests were carried out which demonstrated a constancy in the radiation dosage emitted over a period of days by the x-ray tube.

The LD_{50} was determined for rats maintained on the laboratory stock diet. In the case of adult male and female rats, the LD_{50} was found to be 710 r , while in young adult rats a figure of 625 r was obtained.

RESULTS

Experiments on single exposure to radiation

Table 2 gives the mortality figures, as well as the values for the hemoglobin and leukocyte counts, for male and female rats previously on a fat-free diet or a 30% fat regimen, after having been subjected to 650 or 850 r of irradiation in a single dose (series I). The mortality rate averaged only 14% for the rats receiving the lower dosage of x-irradiation, while 53% of the rats receiving the higher dosage died. A total of 374 rats was used for the tests; these were approximately equally divided between the two dosage levels.

TABLE 2
The mortality, hemoglobin level, and white cell count of male and female rats for 46 days after irradiation with 650 or 850 r in a single dose (diet 1, 0% fat; diet 4, 30% fat)
Series I

DAYS AFTER IRRADI- ATION	MORTALITY, %				HEMOGLOBIN, % ^a				LEUKOCYTES, % STARTING LEVEL ^b			
	Males		Females		Males		Females		Males		Females	
	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4
	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4	Diet 1	Diet 4
650 r (Rats per group: males, diet 1, 48; diet 4, 37; females, diet 1, 50; diet 4, 50).												
0	8	3	4	0	12.7	12.5	12.8	12.8	27	15	13	14
7	17	3	12	8	12.1	11.8	11.9	10.4	38	30	26	31
13	23	3	14	14	9.0 ¹	10.6	14.7	8.7	32	77	35	73
19	23	8	14	14	11.2	9.7	11.5	11.3	35	60	50	83
25	23	8	14	14	12.4	11.7	13.0	12.3	84	73	61	84
31	23	8	14	14	13.4	14.0	13.3	13.9	55	92	76	72
37	23	8	14	14	13.8	12.7	13.3	12.8	65	89	107	91
43	23	8	14	14	15.3	12.9	12.9	13.1	54	84	120	105
46	23	8	14	14	13.8	13.5	12.7	13.5				
850 r (Rats per group: males, diet 1, 49; diet 4, 40; females, diet 1, 50; diet 4, 50).												
0	4	10	2	8	12.7	12.5	12.8	12.8	7	12	7	10
7	36	43	46	28	11.8	12.1	12.1	12.0	12	11	29	19
13	45	55	54	42	7.6 ³	8.3 ³	7.6 ⁵	7.9 ³	46	33	59	39
19	49	55	56	46	9.0 ¹	7.7 ⁵	8.7 ¹	9.1 ²	46	37	63	71
25	51	55	56	46	12.3	8.9 ³	13.3	11.4	41	45	70	69
31	51	55	56	46	14.3	11.1 ¹	13.3	12.2	48	66	76	63
37	51	55	56	46	14.8	12.3	13.7	12.6	46	68	90	90
43	51	59	56	46	13.9	12.8	14.4	13.2	53	73	89	69
46	51	59	56	46	14.0	12.8	13.6	12.4				

^a The superscript numbers indicate the number of rats having a hemoglobin level too low for accurate determination. These are calculated as 7.5%.

^b Starting counts were as follows: males (diet 1), 21,500; males (diet 4), 19,000; females (diet 1), 17,200; females (diet 4), 17,500.

No appreciable differences in mortality rate can be attributed to the diet. Likewise, no differences in susceptibility to x-irradiation were found to be related to sex.

Little effect on blood hemoglobin values was noted by the 7th day after exposure to x-rays; following this period there was a rapid decline, which reached the minimum value between the 13th and the 19th day. There would appear to have been a somewhat greater resistance to a decrease in the hemoglobin level in the rats on the fat-free diet than in those animals on the diets containing fat. The average values of the hemoglobin for the 8 tests, made after exposure to 650 *r*, were 12.6 and 12.1%, respectively, for the male rats on diets 1 and 4, and 12.9 and 12.0%, respectively, for the females on these two diets. In the case of the tests on the higher dosage (850 *r*), the mean hemoglobin values were 12.2 and 10.8% for the males and 12.1 and 11.4% for the females on diets 1 and 4, respectively. These averages are of limited value since, with the method employed, there is no way to estimate the figures for hemoglobin below 7.5%.

In the case of the blood leukocytes, the minimum level was obtained in all cases in the first post-radiation sample; namely, at 7 days. In surviving animals the value slowly returns to normal, although the recovery is not complete at 46 days in the case of the males after 650 *r*, or in either sex after the 850 *r* dosage. The average percentage for the pre-irradiation leukocyte level found over the 46-day period following dosage with x-irradiation was higher in all cases for females than for males. In three of the 4 groups, the recovery based on white cell count would appear to have been slightly better in the case of the rats on the high-fat diet (diet 4). Thus, the mean percentages of the pre-irradiation leukocyte levels found after exposure to 650 *r* were 49 and 65 for the males, and 61 and 69 for the females receiving diets 1 and 4, respectively. In the tests carried out at the higher x-irradiation dosage the comparative figures are 37 and 43 for the males and 60 and 54 for the females on diets 1 and 4, respectively.

*Experiments with repeated sublethal exposure
to x-irradiation*

Although there was no indication that diet plays a role in the protection of rats from a single exposure of x-irradiation at a fairly high level, it was believed that variations in response might be noted if the stress were less severe and were continued over a longer period. The method employed was to administer single sublethal doses of 300 *r* weekly over a 14-week period, omitting irradiation during 6 different weeks, so that a total of 2,400 *r* was given. In series II the rats received diets containing 15 or 30% of fat (diets 3 and 4), in addition to the fat-free regimen (diet 1), while in series III the fat levels chosen were 0, 2 (diet 2) and 30%. In all cases the fat employed was cottonseed oil, which is known to contain about 50% of essential fatty acids. Assuming a daily food consumption of 10 gm of diet, this would mean that approximately 100 mg of linoleate would be consumed per day by the rats receiving diet 2. The intake of linoleate would probably exceed 600 mg per day with diet 3 and 1,000 mg per day with diet 4. The mortality figures for these tests are recorded in tables 3 and 4.

In both series of tests the rats receiving the diets containing fat showed a marked superiority in resistance to x-irradiation compared with those on the fat-free regimen. In series II the beneficial effects were equally pronounced when the diets contained 15 and 30% of fat. No sex differences in susceptibility to x-irradiation, irrespective of diet, were noted in the tests recorded in table 3. The survival time for the males and females on the fat-free diet averaged 34 and 39 days, respectively, compared with values of 70 and 76 days for the rats on diet 3 (15% fat) and 63 and 69 days for the animals on diet 4 (30% fat). The differences in survival time between the control groups and the experimental groups were highly significant in all cases. The increased survival time occurred in spite of the fact that the average exposure to x-irradiation was 40 to 50% greater for the rats on the fat diets than for those on the fat-free regimen. This situa-

TABLE 3

The percentage of mortality of male and female rats on diets containing several levels of fat when subjected repeatedly to sublethal doses of x-irradiation (diet 1, 0% fat; diet 3, 15% fat; diet 4, 30% fat)

Series II

WEEKS AFTER IRRADI- ATION	TOTAL CUMULA- TIVE IRRADI- ATION	MALE RATS ¹		FEMALE RATS ¹	
		Diet 1 (18)	Diet 3 (24)	Diet 1 (17)	Diet 3 (20)
					Diet 4 (20)
1	300	0	0	0	0
2	600	0	0	5.9	5.0
3	900	5.5	8.3	17.7	5.0
4	1,200	33.3	16.7	23.5	15.0
5	1,200	72.4	20.8	47.0	10.0
6	1,200	77.8	20.8	53.0	10.0
7	1,200	94.4	29.2	70.7	20.0
8	1,500	94.4	33.3	82.4	35.0
9	1,500	94.4	33.3	82.4	40.0
10	1,800	94.4	41.7	94.2	40.0
11	1,800	94.4	45.8	100.0	45.0
12	2,100	100.0	50.0	..	60.0
13	2,100	..	62.5	..	55.0
14	2,400	..	83.4	..	85.0
15	2,400	..	95.8	..	100.0
16	2,400	..	100.0
Average day of death ²		34 ± 3.3	70 ± 6.1	39 ± 4.3	76 ± 7.0
			5.22		4.52
Average exposure per rat in r ²		1,235 ± 54	1,800 ± 112	1,270 ± 77	1,950 ± 128
			5.08		4.55
					3.92

¹ The figures in parentheses at the head of the columns represent the number of rats in each group.

² Including the standard error of the mean calculated by the formula, $\sqrt{\text{Ed}^2/n - 1/\sqrt{n}}$.

The boldface figures are the ratios of mean difference (experimental — control) and $\sqrt{(\text{S.E.M.})^2_e + (\text{S.E.M.})^2_c}$. A value in excess of 3.0 indicates significance.

TABLE 4

The percentage of mortality of male and female rats on diets containing several levels of fat when subjected repeatedly to sublethal doses of x-irradiation (diet 1, 0% fat; diet 2, 2% fat; diet 4, 30% fat)

Series III

WEEKS AFTER IRRADI- ATION	TOTAL CUMULA- TIVE IRRADI- ATION	MALE RATS ¹			FEMALE RATS ¹		
		Diet 1 (30)	Diet 2 (30)	Diet 4 (26)	Diet 1 (26)	Diet 2 (30)	Diet 4 (29)
1	300	0	0	0	0	0	0
2	600	0	3.3	0	3.8	0	3.4
3	900	3.3	3.3	0	7.7	0	6.9
4	1,200	10.0	3.3	3.8	11.5	3.3	10.3
5	1,200	20.0	10.0	7.7	11.5	3.3	10.3
6	1,200	33.3	10.0	11.5	11.5	3.3	13.8
7	1,500	43.3	13.3	11.5	15.4	10.0	13.8
8	1,800	56.7	23.3	19.2	23.1	23.3	20.7
9	2,100	73.3	36.7	23.1	53.8	40.0	51.7
10	2,400	90.0	63.3	57.7	73.0	53.3	68.9
11	2,400	93.3	76.7	73.2	84.6	70.0	86.2
12	2,400	100.0	90.0	80.8	88.5	80.0	96.5
Surviving at end		0.0	10.0	19.2	11.5	20.0	3.5
Average day of death ²		51 ± 3.1	65 ± 3.0	67 ± 3.2	61 ± 3.6	67 ± 2.6	62 ± 3.4
Average exposure per rat in r ²		1,770 ± 99	2,205 ± 92	2,290 ± 82	1,980 ± 84	2,170 ± 62	2,080 ± 87
			3.22	4.07		1.82	0.82

¹ The figures in parentheses at the head of the columns represent the number of rats in each group.

² Including the standard error of the mean calculated by the formula, $\sqrt{\sum d^2/n - 1/n}$.

The boldface figures are the ratios of mean difference (experimental - control) and $\sqrt{(\text{S.E.M.})^2 + (\text{S.E.M.})^2}$. A value in excess of 3.0 indicates significance.

tion obtained because irradiation was continued as the experiment progressed, and the rats which survived were obviously exposed to greater doses than had been those animals which succumbed earlier.

In general, the data presented for series III in table 4 confirm the results of series II. However, it is shown that cottonseed oil is almost equally as effective in affording protection from the toxicity of irradiation at a 2% level in the diet as at a 30% level. In contradistinction to the results obtained in series II, a marked sex difference in resistance to x-irradiation was noted on the several diets in series III. The males receiving either diet 2 or 4 had a considerably higher survival rate throughout the test. The average time of death was 51 days for the male rats on the fat-free diet (diet 2), 65 days for the animals on diet 2 (2% fat) and 67 days for those on diet 4 (30% fat). These variations are likewise highly significant.

On the other hand, the variation in the survival time of the female rats in the several groups was slight, the values being 61, 67, and 62 days, respectively. It is apparent that the failure to demonstrate any significant increase in survival time in the females on the fat-containing diets is due to the fact that the females in the control group lived for a longer period than did the animals in the control group which was composed of male rats. Actually, the average number of days of survival on the two fat diets was quite similar in the corresponding male and female groups.

There are several possible explanations for the variations found in series II and III. The animals in series II were approximately 18 months old, while those in series III were young animals less than three months old at the start of the tests. A second difference is the rate at which the x-irradiation was given to the animals. In series II, 14 weeks were required for the administration of 2,400 *r*; no x-irradiation was given between the 4th and the 7th week or during the 9th, 11th, and 13th weeks. In the case of series III, the total

x-irradiation covered a period of only 10 weeks; x-irradiation was omitted only from the 4th to the 6th week.

The data have also been subjected to statistical evaluation by the use of the probit scale or the N.E.D. (normal equivalent deviate) as described by Moore, Cramer and Knowles

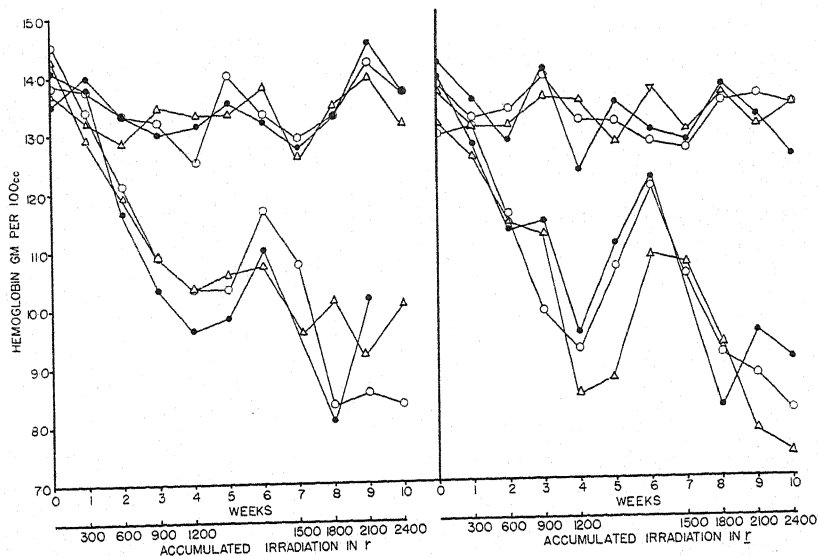


Fig. 1 Average values for hemoglobin in grams per 100 ml of blood of male rats (left) and female rats (right) before and after x-irradiation with 2,400 r over a 10-week period. The upper group of curves represents hemoglobin values for the normal controls, and the lower group of curves that for the irradiated animals. Curves with black circles represent data for animals on a fat-low diet (diet 1); those with open circles present data for rats on a 2% fat diet (diet 2); and those with open triangles give results for rats on a 30% fat diet (diet 4).

('51). On the basis of such an analysis⁵ it was shown that frequency of death, calculated for the 42nd day after all groups had received 1,200 r , with the sexes combined, involved highly significant differences when the results for rats on the fat-free regimen are compared with those for animals on the fat-containing diets. The value for "p" was < 0.001 for

⁵ The authors wish to thank Professor Fred Moore and Mr. Robert Knowles of the Department of Experimental Medicine of the School of Medicine of the University of Southern California for their aid in some of the statistical evaluations.

series II and < 0.02 , > 0.01 for series III. That the age of the animals plays a role is likewise shown by the fact that a highly significant figure is obtained when data on the rats on the fat-free diets in series II and III are compared (< 0.001) or when the frequencies of death of the rats receiving the

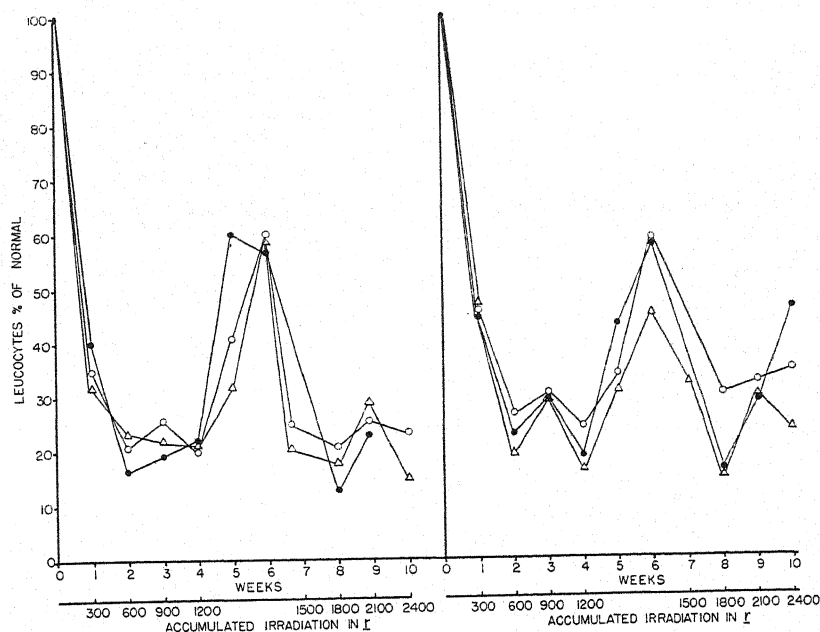


Fig. 2 Average values for leukocyte count in per cent of normal in male rats (left) and female rats (right) after irradiation with 2,400 r over a 10-week period. Curves with black circles represent data for animals on a fat-low diet (diet 1); those with open circles present data for rats on a 2% fat diet (diet 2); and those with open triangles give results for rats on a 30% fat diet (diet 4).

fat diets in the two series are compared ($p = < 0.01$, > 0.001). However, this method of statistical treatment fails to show clear differences between the sexes or between the responses to the several levels of fat.

Figure 1 summarizes the changes in hemoglobin, while figure 2 gives a summary of the variation in leukocyte count, of series III during the 10-week period of the experiment.

As would be expected, the hemoglobin level remained at a subnormal value during the entire experiment. However, in line with the lower dosages of x-irradiation employed, the rate of decrease in hemoglobin was less in the present tests than in either experiment in series I, in which a single exposure to a large dosage was used rather than repeated exposures to sublethal levels of x-irradiation, as occurred in series III. The minimum hemoglobin value was reached during the 4th week and again during the 10th week (4 weeks after the resumption of x-irradiation). No variations in the average level are to be noted in the males, as related to diet. In the case of the females, the results for the group on diet 4 appear to be slightly less satisfactory than those for the animals on diets 1 or 2.

The white cell count, which is the most sensitive index of irradiation injury, remained at the minimum value during the entire 10-week period, except that a partial recovery obtained between the 4th and the 7th week when x-irradiation was suspended.

DISCUSSION

Although the therapeutic effect of a fat diet cannot be demonstrated when exposure to x-irradiation is at a high level, there is no question but that this foodstuff serves to protect rats from repeated sublethal doses of x-irradiation. The protection occurs in spite of the fact that there is apparently no concomitant improvement in hemoglobin level or leukocyte count over that observed in rats receiving a fat-free regimen.

The factor in fat responsible for the protective action against x-irradiation would appear to be the essential fatty acids. This conclusion is based upon two considerations. In the first place, the essential fatty acids are the only known required components of fat not included in the basal diet. In the second place, the fact that protection occurs when cottonseed oil is given at a 2% level in the diet supports this hypothesis. On the basis of an average consumption of 10 gm

of food daily, this would account for an intake of 200 mg of cottonseed oil or 100 mg of essential fatty acids per day. In the tests in series III there was a slightly lower average mortality for the males on the 30% fat diet than for those on the 2% fat regimen. This may possibly be ascribed to the fact that the essential fatty acid intake was at a suboptimum level on diet 2. In fact, our recent results (Deuel et al., '51) indicate that, in male rats, the optimum level for linoleate is at least 200 mg per day or higher. The fact that as effective protection results from the 2% fat diet as from the 30% fat regimen in the case of females may well be a reflection of the markedly lower essential fatty acid requirement for this sex (Deuel and Greenberg, '50; Greenberg and Deuel, '50; Anisfeld et al., '51).

The sex variation in susceptibility to x-irradiation which was noted in series III but not in series I or II may be related to the difference in fat metabolism of the two sexes — a fact which has been repeatedly demonstrated. Although little variation was noted in the mortality levels for male and for female rats on either of the fat-containing diets (diets 2 or 4), the survival rate of the male rats on the fat-free diet was much lower than that of the females on the same dietary regimen. Thus, one can interpret such data as proving that fat has a sparing action on the male rat subjected to x-irradiation, but that this protective action is much less in the case of females.

The reason for the sex differences in resistance to x-irradiation is not evident. It may be related to the lower linoleate requirement mentioned earlier, or to a variation in the intermediary metabolism of fat. Such differences have been reported in the fasting ketonuria of the human subject (Deuel and Gulick, '32) and in that of rats with fatty livers (Deuel et al., '37), as well as in the exogenous ketonuria of rats and guinea pigs (Butts and Deuel, '33). Similar differences have also been noted in the case of carbohydrate, since a more rapid disappearance of glycogen obtains in the female (Deuel et al., '34).

There is no immediate explanation of the fact that fat exerted a protective effect on both males and females in series II, while its effect was noted chiefly in the case of the males in series III. If differences are to be ascribed to variations in fat metabolism, one would expect these to be minimum for the older rats used in series II, while they would be accentuated in the tests on growing rats recorded in series III.

It is believed that the present experiments demonstrate that the essential fatty acids may be related to protection from irradiation injury. Since the water-soluble vitamin intake was at a greater than optimum level and the protein content of the diets was high (25 to 34%), it is difficult to see how our results could be ascribed to variations in these dietary factors. Moreover, inasmuch as the water-soluble vitamins and proteins were present in constant proportions per 100 Cal. of diet, the rats would be expected to receive uniform quantities of these factors, irrespective of the diet consumed. Finally, the fact that beneficial effects in decreasing irradiation injury were noted in the case of rats on the 2% cottonseed oil diets would seem to indicate that a variation in consumption of water-soluble vitamins or proteins is not the explanation for the therapeutic action of fat diets.

Although the evidence seems to indicate that the protective action of fat is direct, one may still postulate that the presence of fat in the diet has facilitated a more satisfactory utilization of some other dietary component. Thus, by this indirect means, the vitamins or proteins would become responsible for the protection from x-irradiation injury. There are at present no data in support of this latter hypothesis.

SUMMARY

No differences were noted in the susceptibility of rats to x-irradiation injury as related to the fat content of the diet when the animals (on the diets for three to 4 weeks) were subjected to 650 or 850 *r* in a single dose.

On the other hand, when rats (on the diets for 12 weeks) were treated with a series of sublethal doses of x-irradiation

at weekly intervals (2,400 *r* over 10 or 14 weeks in 300 *r* doses), the rats receiving fat in their diets were found to be more resistant, and survived over a longer interval than did those on the fat-free regimen.

The protective effect of fat was approximately as satisfactory when cottonseed oil was incorporated in the diet at a 2% level as when it comprised 15 or 30% of the diet. The beneficial action of fat is therefore believed to be related to its content of essential fatty acids.

In one series of tests on young rats (two to three months old), the protective effect of fat was evident only in the male rats and not in the female rats. However, in a test with older rats (18 months), both sexes were protected to an equal extent by the incorporation of fat in the diet.

In spite of the beneficial results produced by fat in lowering the mortality rate of rats following exposure to x-irradiation, no concomitant improvements were noted in such indices of x-irradiation injury as hemoglobin level or leukocyte count.

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INTRAVENOUS INJECTION OF FAT-SOLUBLE VITAMINS AND TRANSFER TO MILK OF INJECTED VITAMIN A¹

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THREE FIGURES

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The purpose of this investigation was to study the transfer to milk of vitamin A dispersions injected intravenously as compared with that following oral doses of oily and aqueous vitamin A.

Previous studies have indicated that mammary transfer of vitamin A is a function of serum level (Sobel et al., '50). From this it should follow that maximal transfer of vitamin A should be produced by injecting a given dose intravenously, since the highest blood levels are produced in this manner. For this reason, intravenous injections would be of value when oral feeding is not practical, and of marked advantage in favoring diffusion across impaired body membranes by providing high concentration gradients from the blood to a given tissue. Moreover, in animal husbandry, intravenous injections would be a more certain method than oral feeding in overcoming the fat-soluble vitamin deficiencies occurring in ruminants and horses (Oser, '48; Harris, '49).

Since the dispersing agent in aqueous dispersions (Sobel et al., '48) causes hemolysis (Krantz et al., '48; Sobel et al., '49), we determined the maximum concentrations of the dispersing agent tolerated without hemolysis.

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EXPERIMENT ON HEMOLYTIC ACTION OF
DISPERSING AGENT

Ten grams of sorethytan laurate and 1.25 gm of oily vitamin A concentrate, dispersed in 100 ml of 0.85% NaCl solution, were added in increasing amounts to 0.5 ml of heparinized whole blood and the mixture was then incubated at 37°C. for 24 hours. Dilution to 10.0 ml was made with Ringer's solution and this was followed by separation of the intact red cells by centrifugation. One milliliter of the supernatant layer was diluted to 10.0 ml with distilled water.

TABLE 1

Per cent hemolysis of heparinized whole rabbit blood after incubation at 37°C. for 24 hours with sorethytan laurate plus vitamin A concentrate

ANIMAL	CONCENTRATION OF SORETHYTAN LAURATE					
	0 mg %	200 mg %	300 mg %	400 mg %	600 mg %	800 mg %
Rabbit no. 1	0	0	2	18	26	49
Rabbit no. 2	0	0	2	12	27	51
Rabbit no. 3	0	0	2	11	29	55
Rabbit no. 4	0	0	3	15	31	61
Rabbit no. 5	0	0	4	15	34	57
Rabbit no. 6	0	0	2	9	29	58
Mean	0	0	3	14	29	56

Optical density was then read at 548 m μ in a Coleman Spectrophotometer. Conversion into per cent hemolysis was made through calibration curves prepared by hemolyzing 1.0 ml of each blood specimen with 19.0 ml of distilled water and diluting 0.1, 0.2, 0.3, and 1.0 ml aliquots to 10.0 ml with distilled water (10, 20, 30, and 100% hemolysis, respectively). The results are shown in table 1. The red cells in 100 ml of heparinized whole blood resisted as much as 200 mg of sorethytan laurate, and with 300 mg, only 2 to 4% hemolysis occurred.

Once the above conditions were established, we prepared and administered aqueous dispersions in such a manner that only 30 to 40 mg of the dispersing agent were present in 100 ml of blood, thus allowing a wide safety margin.

TOXICITY OF AQUEOUS DISPERSIONS OF
FAT-SOLUBLE VITAMINS

After receiving 6 injections of 400 μ g of vitamin A per pound of body weight, 4 of the test animals (rabbits) were sacrificed. Gross examination and microscopic survey of vital organ sections revealed no pathology, nor was any hemolysis observed in the blood. An examination of serum for total lipid content, cholesterol esters (Zuckerman and Natelson, '48), free cholesterol (Sobel and Mayer, '45), and urea (Sobel and Hirschman, '47), and of liver for vitamin

TABLE 2
Post-injection analyses of rabbit tissues

RABBIT	BODY WEIGHT	TOTAL LIPIDS ¹	TOTAL CHOL. ¹	FREE CHOL. ¹	FREE CHOL. ¹	UREA ¹	LIVER VIT. A	HEMATO- CRIT
	lb.	mg %	mg %	mg %	%	mg %	μ g	
1	8.2	223	106	29.8	28.1	19.5	1690	0.31
2	8.8	187	84.5	21.4	25.3	16.0	1740	0.29
3	10.8	200	84.5	21.4	25.3	15.8	2440	0.32
4	9.8	503	297	76.4	25.7	..	7820	0.34
Mean	9.4	278	143	37.2	26.1	17.1	3423	0.32

¹ Serum.

A (Sobel et al., '48) revealed no pathology (table 2). An additional 32 rabbits receiving injections appeared in good health. There were no deaths, whereas control animals died from random infections during this period.

VITAMIN A TRANSFER TO MILK FOLLOWING
INJECTION OF VITAMIN A

It became evident that the production of high blood levels of fat-soluble vitamins by intravenous injection of aqueous dispersions is practical. We next proceeded to compare mammary transfer following intravenous injection of an aqueous vitamin A dispersion with that following oral administration of the same vitamin A product.

Five dairy cows received by jugular vein 0.1 ml per pound of body weight of a dispersion containing 230 μ g of vitamin A per 0.1 ml 0.85% NaCl. Blood was collected prior to

the injection followed by specimens three, 30 and 60 minutes after the injection. All of the injected vitamin was recovered in the three-minute post-injection blood sample. Milk was collected for 24 hours prior to the dose and 48 hours thereafter, and examined for vitamin A content (Sobel and

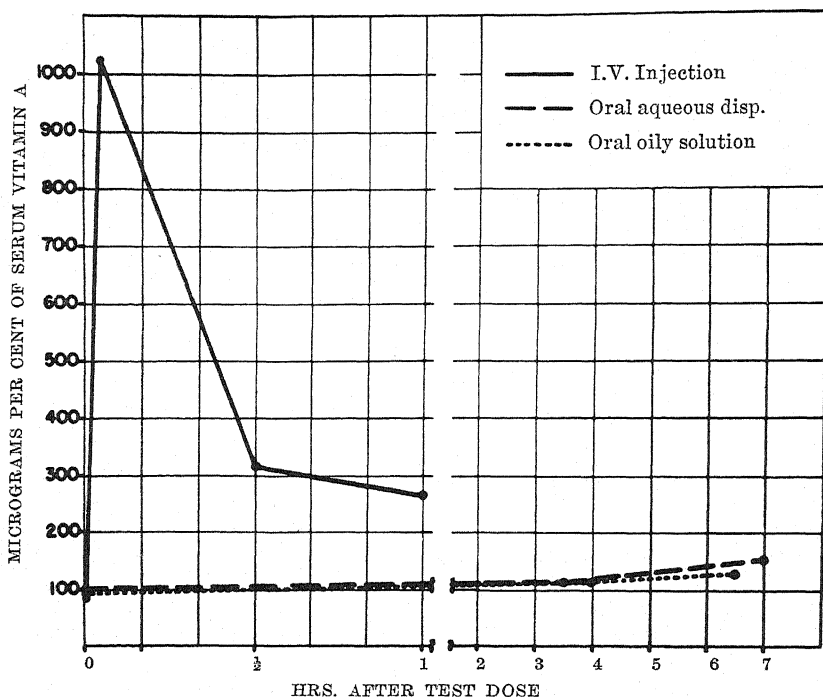


Fig. 1 Comparison of serum vitamin A levels in cows after administration of test dose by intravenous injection, aqueous dispersion orally, and oily solution orally.

Rosenberg, '49). The same dose was given orally to three additional cows by quantitative rinsing through a stomach tube. (One of the three cows given aqueous dispersions orally was eliminated from this series because there was no change in milk levels during the 50 hours after the test dose.) Blood was collected prior to the dose and also three and one-half, 4, and 7 hours thereafter, and the samples were analyzed for vitamin A (Sobel and Snow, '47). The same method of collect-

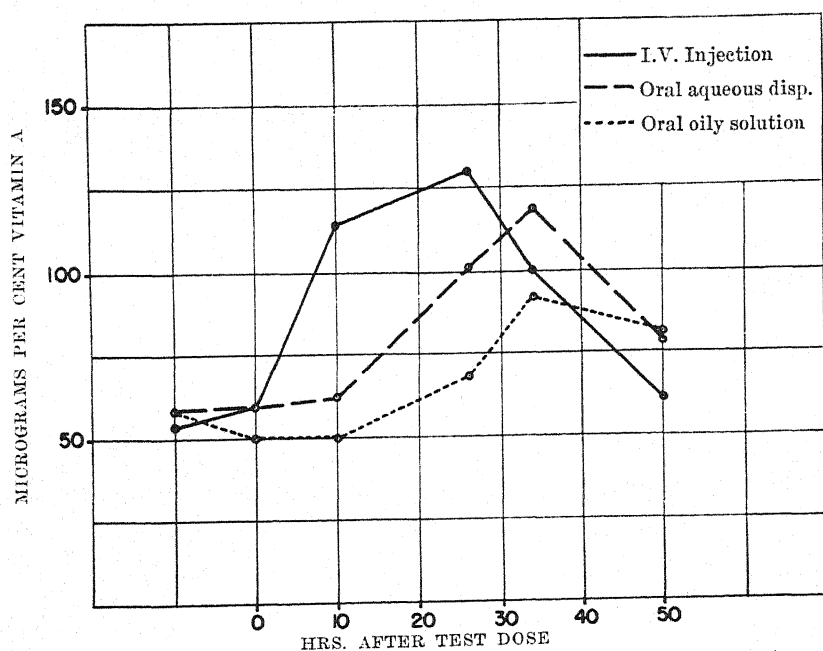


Fig. 2 Comparison of milk vitamin A levels in cows after administration of test dose by intravenous injection, aqueous dispersion orally, and oily solution orally (mean values).

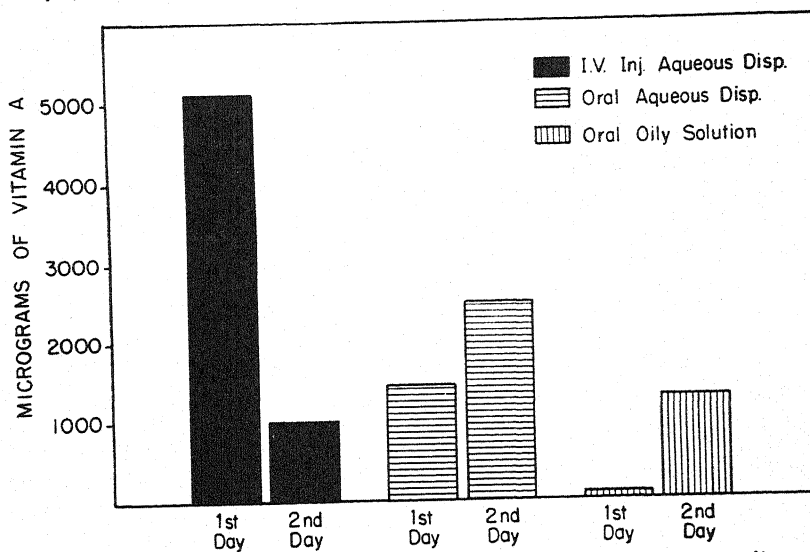


Fig. 3 Comparison of increase in milk vitamin A secretion in cows after administration of test dose by intravenous injection, aqueous dispersion orally, and oily solution orally.

ing milk was used. As a further comparison, three additional cows received the same vitamin A dissolved in corn oil. Blood was collected before the dose and again three and one-half and 6½ hours following the dose. Results are compared in figures 1, 2, and 3. They indicate that intravenous injections are far more effective in producing greater vitamin A transfer from blood to milk, and do so in a shorter time than when oral administration is made of either aqueous dispersion or oily solution. Transfer with the aqueous dispersion given orally is greater and occurs sooner than with the oily solution. The mean volume of milk secreted in liters per cow is given in table 3.

TABLE 3
Amount of milk secreted (mean) in liters per cow

HOURS	HOURS BEFORE TEST DOSE		HOURS AFTER TEST DOSE			
	10	0	10	26	34	50
Intravenous injection	3.6	4.3	3.3	4.8	3.3	4.3
Oral aqueous	3.5	2.5	2.8	3.8	2.5	3.9
Oral oily	2.5	2.0	1.9	2.6	2.4	2.3

DISCUSSION

The mechanisms that regulate the composition of milk are still obscure. One of the limiting factors that can be postulated is the transport to the site of milk production of those components of milk that are not manufactured locally. Theoretically, the diffusion of a substance like vitamin A from the blood across a membrane would be proportional to the concentration gradient of the diffusing substance.

The approximate computation is $A = KC$ where A = amount of vitamin A diffusing through a unit area per unit time; C = difference in concentration of vitamin A on the two sides of a given membrane, i.e., serum vitamin A and filtrate across capillaries; and K = constant depending upon type and thickness of membrane. A more exact presentation is given by Davson and Danielli ('43).

Our previous studies in humans indicate that such considerations apply to vitamin A transfer from blood to milk (Sobel et al., '50). When higher blood levels were obtained milk levels followed. Since blood levels are maximal following intravenous injection (fig. 1), milk levels should be higher and reach an earlier maximum than with orally ingested aqueous dispersions. This was so when milch cows received (1) intravenous injections of vitamin A, (2) oral doses of the same dispersion, and (3) oral doses of the same vitamin A in oil. The increase in milk vitamin A for 24 hours after the dose was given was 15 times as great following the injection, and was 5 times as great with oral doses of the aqueous dispersion as with oily solutions given orally. The maximum, following the theory, took place much earlier with intravenous injection than with oral administration (figs. 2 and 3). This marked increase in transfer of vitamin A is important where deficiency may exist in one or more tissues due to impaired membranes of cell and capillary walls. Intestinal membrane offers an analogy. Vitamin A deficiency may result when this membrane is defective or injured, or the chemical system necessary for fat absorption is not functioning (May and Lowe, '48; Popper et al., '47; Kramer et al., '47). A similar situation may exist in fat-soluble vitamin transfer from blood to a tissue. This may be the reason why a variety of skin diseases displaying hyperkeratosis improve after prolonged treatment with high vitamin A doses, which provide the high blood levels probably needed to overcome resistance of impaired membrane systems, and allow a sufficient amount of the vitamin to reach the affected tissue (Harris, '49). In acne vulgaris, earlier improvement was obtained with the high blood levels following aqueous dispersions (Davidson and Sobel, '49) than reported by Straumfjord ('43) who gave twice the dosage in oil. Intravenous injections, by-passing possible intestinal absorption barriers, produce immediate maximal blood levels of controlled concentration, and should prove highly effective in

the treatment of local tissue deficiency of the fat-soluble vitamins.

A great deal of evidence in the recent literature indicates that the lack of sufficient fat-soluble vitamins, especially of vitamin A, is a problem not only in human nutrition, but also in animal husbandry in raising ruminants and horses (Harris, '49; Oser, '48). The existence of vitamin A deficiency in dairy cows on normal rations has been emphasized by Tom ('47) and again by Alvarez ('47), who noted corneal ulceration and interference with gestation due to vitamin A deficiency. Madsen and Earle ('47) reported the condemnation of 651 beef carcasses for edema due to vitamin A deficiency. Madsen et al. ('48) further reported that fertility in bulls is established only when sufficient vitamin A is received. In the absence of this supplement, rapid decline of sperm motility and sexual activity follows. Sterility in horses was overcome by the parenteral administration of vitamin A (Ribeiro and Janz, '46). Scours is a disease of newborn calves in which vitamin A deficiency is a factor. Since the young calf receives its vitamin A from the mother's milk, the remedy lies essentially in increasing the vitamin A content of the milk secreted (Oser, '48; Wise et al., '46). Administration of vitamin A by mouth fails due to the distinct impairment of vitamin A absorption that exists in calves with scours (Sellers et al., '49). The intravenous administration of the fat-soluble vitamins may be most practical for the large ruminants, since it would assure that the animal actually obtains the complete dose and conditions for transfer to milk and to those organs of the body which need these vitamins is maximal.

SUMMARY

Milch cows were given (1) intravenous injections of an aqueous vitamin A dispersion, (2) the same dispersion orally, and (3) the same vitamin A dissolved in corn oil. Increased milk vitamin A following the dose was 15 times as great with intravenous injection and 5 times as great with an oral

dose of the aqueous dispersion as with an oily solution given orally (oral doses were given through a stomach tube). The maximum transfer took place much earlier with intravenous injection than with oral administration. This is in harmony with the theory of diffusion, since maximum blood levels are reached immediately and are higher following intravenous injections than with oral doses. After repeated injection of the aqueous dispersion into rabbits, gross examination, microscopic survey of vital organ sections, and chemical analysis revealed no pathology.

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DIMINISHED URINARY CREATININE IN VITAMIN E DEFICIENT RATS¹

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The urinary excretion of creatinine by rabbits deficient in vitamin E was shown to be unchanged, except in terminal stages, by Mackenzie and McCollum ('40), while creatine excretion rose sharply. These findings relative to the creatinine and creatine excretion by rabbits have been confirmed many times. However, some evidence has been obtained (Hove, '49) that rats deficient in vitamin E show a somewhat different excretion pattern, in that creatinine diminishes to nearly the same degree as the creatine increases. Data to confirm and extend this observation are presented in this paper.

EXPERIMENTAL

Male rats of Sprague-Dawley origin were reared from weaning (average body weight 48 gm) on diets composed of: water-washed casein (Salmon, '47) at 10% or 18%; salt mixture (Salmon, '47), 4%; vitamin pre-mix, 10%; lard, 9%; cod liver oil, 1%; and sucrose to 100%. The vitamin pre-mix contained pure vitamins mixed with sucrose to furnish the following levels per gram of diet: thiamine, riboflavin and pyridoxine,

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5 µg each; calcium pantothenate, 25 µg; niacin, 50 µg; choline, 2 mg; *i*-inositol, 0.2 mg; and 2-methyl 1-4 naphthoquinone, 0.5 µg. Approximately half of the rats in each of the several groups received the diets to which 0.01% DL- α -tocopherol acetate had been added. The animals were housed individually and fed ad libitum.

TABLE 1
Diminished creatinine excretion by vitamin E-deficient rats

CASEIN LEVEL IN THE DIET	TIME	NO. OF RATS	AVER. BODY WT.	DIETARY VITAMIN E (0.01%)	URINE CREATINE	URINE CREATININE ¹			TOTAL AS CREA- TININE ²
					Aver.	Aver.	S.E.	t	
					<i>mg per day per kg of body weight</i>				
18	2	6	230	—	4.2	22.4			26.0
	2	4	235	+	2.6	24.8			27.0
	8	14	391	—	14.3	19.8	0.93		32.1
	8	12	461	+	4.2	26.3	1.26	4.16	30.0
	20	16	336	—	16.6	19.0	0.95		33.3
	20	14	437	+	3.1	24.1	0.55	4.63	26.8
	1	5	101	—	9.4	31.8			39.9
	1	5	110	+	4.3	32.3			36.0
10	2	10	145	—	22.0	23.1	1.32		42.1
	2	10	164	+	5.6	29.5	2.08	2.60	34.3
	4	15	215	—	14.9	18.7	0.76		31.5
	4	14	227	+	2.4	28.6	0.90	8.40	30.7

¹ Standard error of the mean (S.E.) was derived from the formula: $S.E. = \sqrt{S^2 d^2 / n(n-1)}$.

The value of *t* was derived from the formula: $t = \frac{D}{\sqrt{SE_1^2 + SE_2^2}}$.

² The total included the creatinine plus the creatine/1.16.

At the time intervals indicated in table 1, 24-hour urine samples were collected and diluted to 100 ml. Creatinine was determined by the alkaline picrate method as outlined by Hawk, Oser, and Summerson ('49). Creatine was determined by the direct method of Ennor and Stocken ('48).

The results given in table 1 show that the development of a vitamin E deficiency in rats, as indicated by the increased creatine excretion, was associated with a highly significant

decrease in creatinine excretion, and with a tendency for the total of these two components, expressed as creatinine, to be constant. All of the rats on the 18% casein diet deficient in vitamin E for 20 months showed signs of muscular dystrophy on gross examination. Three of the rats had severe dystrophy with the entire hind-quarters functionless, 9 had moderately severe dystrophy, while the remaining 4 had only slight dystrophy as shown by laxness in the hind legs after manual extension. None of the other animals reported in the table showed gross evidence of dystrophy.

Rats on the diet with 10% casein developed a marked creatinurea and a concomitant decline in creatinine excretion by the end of two months, while at this time the animals on the higher casein diet showed little, if any, evidence of deranged creatine metabolism. These results reaffirm the importance of the casein level on the development of vitamin E deficiency symptoms. An influence of the dietary casein level on the vitamin E requirement of rats was shown by Hove ('46) and confirmed by Hove and Harris ('47), Moore ('49), and Granados, Aaes-Jorgensen and Dam³ ('50).

In table 2 are given the correlation coefficients between creatine and creatinine for the major groups of animals studied. For 55 animals deficient in vitamin E the correlation coefficient r was -0.36 between urinary creatinine and creatine. This correlation is significant at the 1% confidence level, and indicates that the analytical variations within this group reflect different degrees of the deficiency state of the animals. On the other hand, the coefficient of correlation between urinary creatine and creatinine of the 50 rats receiving vitamin E was -0.19 . This falls short of statistical significance and indicates that the variations in the analytical values may be attributed to analytical error or random biological varia-

³ These authors apparently were under the impression that Hove ('46) reported complete protection against all symptoms of a vitamin E deficiency when the casein level was raised to 40%. While this level of casein did prevent depigmentation (i.e., complete whitening) of the upper incisors, it is immediately evident from the data that even this level did not prevent partial de-colorization.

bility. When the groups with and without vitamin E were combined and the coefficient of correlation between creatine and creatinine of all 105 rats calculated, the resulting r was -0.59 . This result seemed mathematically impossible and

TABLE 2
Correlation coefficients between creatinine and creatine excretion values of the rats included in table 1¹

GROUP	NO. OF RATS	CORRELATION COEFFICIENT ² BETWEEN CREATINE AND CREATININE	VALUE OF t NECESSARY FOR SIGNIFICANCE AT THE 1% PT. ³
All vitamin E deficient rats	55	-0.36	0.354
All rats with vitamin E	50	-0.19	0.372
All rats	105	-0.59	0.254

¹ The small groups at one month on the 10% casein diet and two months on the 18% casein diet were omitted from these calculations.

² Calculated from the formula $r = S_{xy}/S_x \cdot S_y$ where x and y are the deviations from the means.

³ From page 149 of *Statistical Methods*, 4th edition, 1946, by G. W. Snedecor, The Iowa State College Press, Ames.

TABLE 3
Muscle and urine creatine of rats on 10% casein and 18% casein diets (Each group had 7 male rats maintained on the diets for 8 weeks from weaning)

DIETARY LEVEL		AVER. WEIGHT GAIN IN 8 WKS.	AVER. MUSCLE CREATINE (dry basis)	AVER. URINE CREATINE DAILY
Casein	α -Tocopherol			
%	%		mg/gm	mg/kg
10	0.01	120	17.8 ± 0.66 ¹	5.4
18	0.01	171	15.3 ± 0.54	2.3
10	0	102	15.4 ± 0.80	20.9
18	0	177	14.1 ± 0.46	2.8

¹ Standard error of the mean.

therefore was adequately checked. However, it reflects merely the wide separation between the analytical values in the two groups of animals.

The seemingly higher average creatinine values of all of the rats on the 10% casein diet with vitamin E (30.1 mg/day/kg) as compared with the larger rats on the 18% casein

diet with vitamin E (25.3 mg/day/kg) (table 1) was reflected in a similar variation in muscle creatine. Rats from another experiment after 8 weeks on the diet showed the results given in table 3. Muscle creatine was significantly higher in rats on the lower casein diet; this may account for the higher creatinine excretion. The reason for the higher muscle creatine in such rats is not clear. It may simply reflect a lesser concentration of muscle cells in the larger animals due to greater quantities of intramuscular fat and fibrous tissue.

DISCUSSION

The relative immediacy of the need for vitamin E by the growing rat and rabbit probably relates to different metabolic processes in these species. It was not surprising, therefore, to note a different pattern in the excretion of creatine and creatinine.

The data indicate that in the vitamin E deficiency in rats the creatinine excretion decreased significantly. This decrease in creatinine excretion correlated negatively with the increased excretion of creatine and tended to minimize the total loss of creatine plus creatinine. It should be noted that the diet contained no added folacin, vitamin B₁₂, sulfur amino acids, or yeast. These are involved in some way with vitamin E metabolism, possibly through creatine synthesis in the liver of the animal. The growth increment in rats due to vitamin E additions to a low-protein diet can be replaced by vitamin B₁₂, especially when combined with folacin (Hove and Hardin, '51b), or by a yeast preparation or xanthine (Hove and Harris, '47). Gyorgy and Goldblatt ('49) state that sulfur amino acids replace vitamin E in protecting rats against fatal liver necrosis. Hardin and Hove ('51) reported that complete protection against methionine toxicity in young rats was prevented only when vitamin E and folacin were included in the diet along with glycine and arginine. Conversely, glycine toxicity was prevented by a combination of methionine and vitamin E. These facts support previous evidence

of the involvement of vitamin E in creatine synthesis (Hove and Hardin, '51a).

A diminished creatinine excretion in progressive muscular dystrophy of humans was emphasized by Hoagland et al. ('45). These workers noted that the decreased creatinine excretion counterbalanced the increased creatine excretion in this condition. However, the dystrophy in humans cannot be cured by simple vitamin E therapy. Scott ('51) reported studies on the enlarged hock disorder in turkeys, a condition said to resemble muscular dystrophy in other animals and strongly suspected of involving vitamin E in some way. While vitamin E was only slightly protective against this disease, the data do show that creatinine excretion in the absence of vitamin E was low. The average value, without vitamin E in the diet, was 10.8 mg/day/kg, and with vitamin E supplements it was 19.2 mg/day/kg.

The data presented herein seem consistent with the hypothesis that creatinine excretion diminishes in those species of animals in which the vitamin E deficiency assumes a chronic state but does not change in animals such as the rabbit in which the need for vitamin E is so urgent that death ensues after only a few weeks of its absence from the diet.

SUMMARY

Rats on diets deficient in vitamin E excreted less creatinine than did control rats receiving this factor. The diminished creatinine excretion correlated significantly with the increased creatine excretion of the deficient animals.

On the 10% casein diet, without vitamin E, rats developed the low creatinine-high creatine pattern much sooner than rats on the 18% casein diet.

ACKNOWLEDGMENT

Appreciation is expressed to Dr. Keith Gregory of this department for checking the calculation of correlation coefficient.

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THE EFFECT OF VITAMIN B₁₂ UPON THE UTILIZATION OF CHOLINE AND BETAINES BY THE YOUNG POULT

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TWO FIGURES

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The relationship between vitamin B₁₂ and methylating compounds in the chick has been demonstrated by Schaefer et al. ('49) and Gillis and Norris ('49). The latter workers also found that chicks deficient in vitamin B₁₂ utilized betaine more effectively than choline, while normal chicks used either supplement equally well for growth when a diet of practical feedstuffs was employed (Gillis and Norris, '51a). Jukes and Stokstad ('51) reported that with the use of a purified diet, the choline requirement for growth was reduced by supplementing the ration with vitamin B₁₂, while the choline required to prevent perosis was not decreased.

Since the poult requirement for choline is greater than that of the chick (Jukes, '40; Evans et al., '42), it was of interest to determine the effect of vitamin B₁₂ upon the utilization of choline and betaine by the poult.

EXPERIMENTAL

Three experiments were conducted in which poults were fed rations containing various combinations of vitamin B₁₂, choline chloride and betaine hydrochloride. The basal ration contained 55% of soybean oil meal which had been extracted with methanol. The remainder of the ration comprised calcium gluconate, 5.0%; calcium phosphate (tribasic), 3.0%;

cottonseed oil, 2.5%; salt mixture (Kratzer et al., '49), 2.5%; vitamin mixture¹ (Kratzer et al., '49), 2.0%; calcium carbonate, 1.5%; fish oil (2,250 A, 300 D), 1.0%; alpha-tocopherol acetate, 0.002% and cornstarch to total 100%.

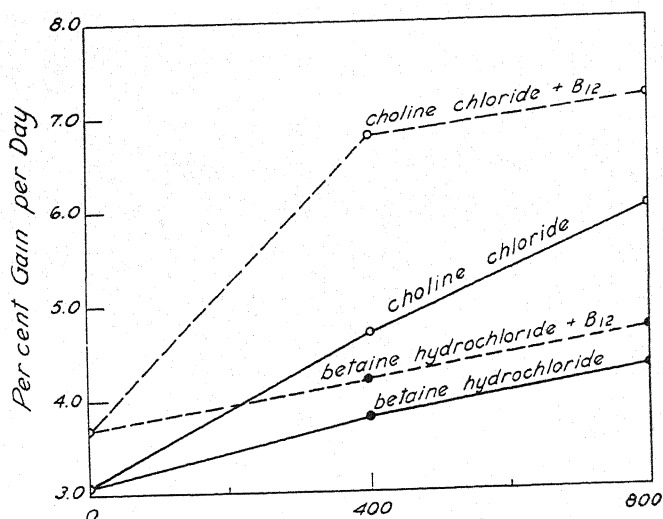
Crystalline vitamin B₁₂² in water solution was added as a supplement to some of the rations (20 µg/kg) and each poult on a vitamin B₁₂-supplemented ration was also given an injection of 10 µg of vitamin B₁₂ into the breast muscle at the start of the experiment. Pure betaine hydrochloride² was used as a supplement, while the choline chloride used was a commercial product which was mixed with calcium carbonate. The amount of calcium in the ration was maintained constant by varying the amount of calcium carbonate to compensate for that added with the choline chloride. By calculation (Almquist, '48), the ration contained 0.47% methionine, 0.40% cystine and 26.6% crude protein. The basal ration was thus somewhat deficient in methionine, since the poult requires approximately 0.5% (Kratzer et al., '49) in a ration containing 24% protein.

Bronze poults from hens which were depleted of vitamin B₁₂ (Kratzer, '52a) were used in the first 5 groups of experiment 1 and throughout experiment 2. Poults from bronze hens fed a normal breeding ration were used for groups 6 to 10 in experiment 1 and throughout experiment 3. The poults for experiments 1 and 2 were fed a vitamin B₁₂-deficient ration for 9 days and 6 days, respectively, before they were divided into equivalent groups and fed the experimental rations. In experiment 3 the day-old poults were placed on experiment immediately without being fed a depletion ration. The three experiments were continued for 13, 15 and 19 days successively. The poults were weighed at frequent intervals and the incidence of perosis, characterized by a bending and twisting of the shank, was noted.

¹ The pteroylglutamic acid was kindly supplied by Lederle Laboratories, Inc., Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

² Kindly supplied by Merck and Co., Rahway, New Jersey, through the courtesy of Dr. D. F. Green.

The basal rations used in each experiment were assayed for choline by the method of Horowitz and Beadle ('43), employing *Neurospora crassa*. The basal rations were found to contain 6.2, 6.2 and 4.9 mg/100 gm in the successive experiments.



Milligrams of Choline Chloride or its Molar Equivalent of Betaine Hydrochloride Added per Pound of Ration.

Fig. 1 Effect of choline chloride, betaine hydrochloride and vitamin B₁₂ upon the growth of poult chicks deficient in vitamin B₁₂.

RESULTS

Reasonably good agreement was noted in the gains of the poult chicks in experiments 1 and 2, and the average percentage³ gains of these two experiments are plotted in figure 1. The addition of choline chloride gave a straight line growth response at the levels used. When vitamin B₁₂ was also added, growth increased at all levels of choline but was much greater at the 400-mg level. This indicates a sparing action of vitamin B₁₂ upon the choline required for the growth of poult chicks.

³ Per cent gain per day = $\left(\frac{\text{Ave. gain} \times 100}{\text{Ave. wt.} \times \text{no. days}} \right)$.

Betaine hydrochloride gave only a slight growth increase at either level, and the groups receiving vitamin B₁₂ gained slightly more than the controls. There was little response to vitamin B₁₂ at various levels of choline when poult from normal hens were used in experiment 3 (fig. 2).

The incidence of perosis decreased as the choline in the ration was increased (table 1) but at each level the addition

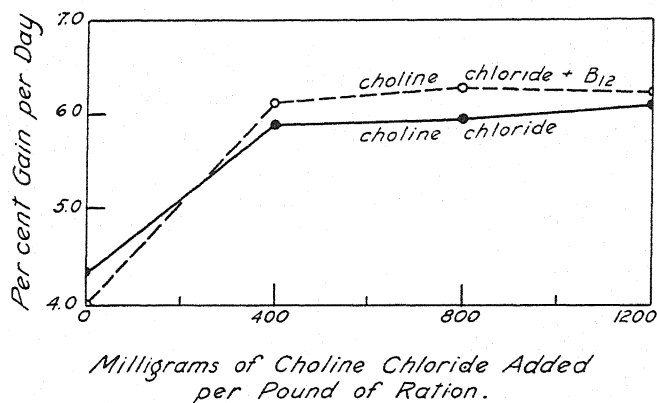


Fig. 2 Effect of choline chloride and vitamin B₁₂ upon the growth of poult with normal stores of vitamin B₁₂.

TABLE 1

Effect of choline, betaine and vitamin B₁₂ upon the incidence of perosis in turkey poult

SUPPLEMENT			INCIDENCE OF PEROSIS		
Choline chloride	Betaine hydrochloride	Vitamin B ₁₂	Expt. 1	Expt. 2	Average (of survivors)
mg/lb.	mg/lb.		%	%	%
0	0	0	33	67	56
400	0	0	0	25	13
800	0	0	0	0	0
0	400	0	20	63	46
0	800	0	100	92	95
0	0	+	90	64	76
400	0	+	80	42	59
800	0	+	40	0	19
0	400	+	100	100	100
0	800	+	100	100	100
Stock mash			10		

of vitamin B₁₂ caused an increase in perosis. Betaine also appeared to increase the incidence of perosis, since the highest level gave a 95% incidence. The addition of vitamin B₁₂ to the diets of the groups fed betaine resulted in a 100% incidence of perosis.

DISCUSSION

The results indicate that vitamin B₁₂ can spare the amount of choline required in the ration of poults to give optimum growth but that it increases the level needed for the prevention of perosis. Other functions of B₁₂ are also indicated by the fact that growth responses were greater with vitamin B₁₂ at all levels of choline and betaine than were observed with control birds.

The sparing action of vitamin B₁₂ upon the requirement for choline for growth in poults agrees with the results obtained with chicks (Gillis and Norris, '51a; Jukes and Stokstad, '51) showing that birds which were deficient in vitamin B₁₂ used choline less efficiently for growth. Gillis and Norris ('51a) discussed the possibility that vitamin B₁₂ is needed for choline oxidase activity to convert choline to betaine, which can then be further metabolized. The increased oxidation of choline as a result of supplying vitamin B₁₂ could presumably reduce the amount of choline available for the prevention of perosis. Gillis and Young ('51), however, were unable to find any difference in the choline oxidase activity of the livers of vitamin B₁₂-deficient and normal chicks.

Gillis and Norris ('51b) have suggested that vitamin B₁₂ is necessary for the synthesis of methyl groups in chicks, as has been shown to be the case with rats. Unpublished results obtained in this laboratory indicate that vitamin B₁₂ is also necessary for methyl synthesis in poults (Kratzer, '52b). The fact that both vitamin B₁₂ and betaine increased the incidence of perosis might be explained by the increase in available methyl groups, although the reason for this effect is not apparent. Scott ('50) has noted an increase in perosis when betaine was added to a ration low in choline. The fact

that vitamin B₁₂ caused growth greater than that of the vitamin B₁₂-deficient groups at all levels of choline and betaine indicates that there are functions of vitamin B₁₂ other than its possible use in methyl synthesis. It is not possible at present to rule out the possibility that the effect of vitamin B₁₂ upon growth may reside in its causing a greater use of choline for growth, thereby decreasing the amount available for prevention of perosis. This does not explain the increase in perosis caused by betaine, however.

The fact that poult from hens fed a normal ration show little response to the addition of vitamin B₁₂ to their ration emphasizes the importance of using poult from hens deficient in vitamin B₁₂ for studies in which vitamin B₁₂ is a variable.

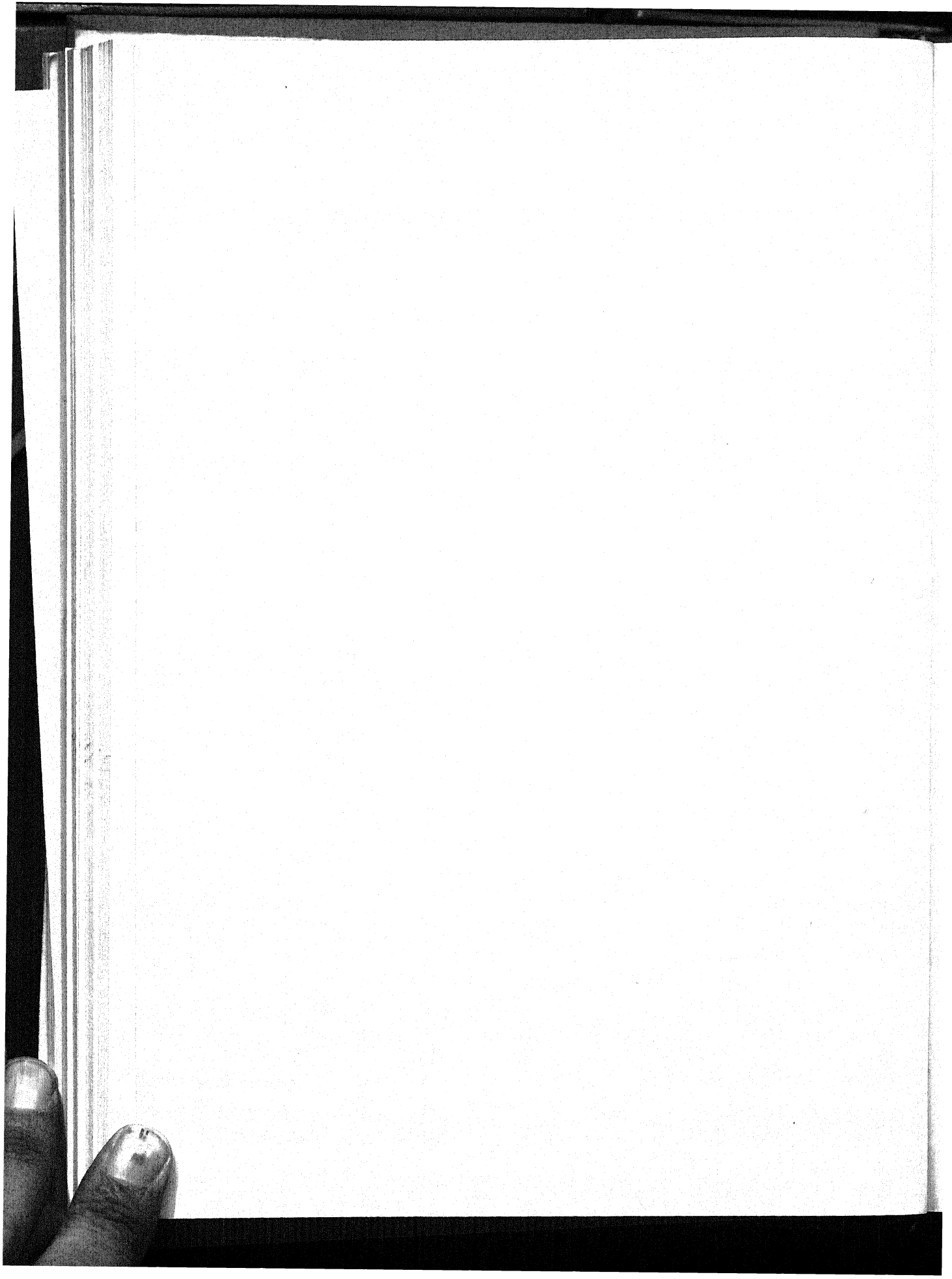
SUMMARY

Poult hatched from hens deficient in vitamin B₁₂ were fed rations containing various amounts of choline, betaine and vitamin B₁₂. Vitamin B₁₂ increased the effectiveness of choline in promoting growth but decreased its effectiveness in preventing perosis. Betaine caused only a slight growth increase on a low-choline ration. Betaine was ineffective in preventing perosis on a low-choline ration.

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FURTHER OBSERVATIONS ON THE UTILIZATION OF HOMOCYSTINE, CHOLINE AND RELATED COMPOUNDS BY CHICKS

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ONE FIGURE

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The effects of vitamin B₁₂ and pteroylglutamic acid (PGA) upon the biological formation and metabolism of choline, betaine and methionine have been the subject of many recent investigations. The experimental approaches used in studying the interrelationships in this group of compounds have been diverse. Some investigators have used isotopes in work with tissue preparations or with intact rats, and others have followed the growth of rats or chicks. In a preliminary report (Jukes et al., '50) it was noted that the growth of vitamin B₁₂-deficient chicks was increased by methionine but not by homocystine with or without betaine. When vitamin B₁₂ was supplied, growth responses were obtained to methionine, homocystine or homocystine plus betaine, thus indicating a role for vitamin B₁₂ in the formation of methionine from homocystine by chicks. In the present investigation it was found that growth was usually produced in B₁₂-deficient chicks by adding a mixture of betaine or choline with homocystine to the B₁₂-deficient purified diet, but the response was no greater than that obtained with betaine or choline alone. Only when vitamin B₁₂ was added was there an indication of an interaction between the "methylating" compounds and homocystine as shown by chick growth.

It has been shown that chicks and turkeys, in contrast to rats, are unable to use methionine, betaine or aminoethanol

alone or together as effective substitutes for choline in diets which are deficient in this substance (Jukes, '40, '41; Schaefer et al., '51). Mono- or dimethylaminoethanol can substitute for choline in promoting growth and preventing perosis in chicks on choline-deficient diets (Jukes et al., '45; Schaefer et al., '51), presumably by undergoing biological "methylation" to form choline. By including dimethylaminoethanol in a purified basal diet for chicks, it was possible to study the growth-promoting effects of various substances which could function as "methyl donors"; furthermore, the addition of dimethylaminoethanol enabled the effect of choline to be measured as a source of methyl groups independently of the chick's requirement for the dimethylaminoethanol moiety of the choline molecule. For example, the effect of choline on growth with a methionine-deficient diet containing homocystine was presumably an indication of the extent to which choline methylated homocystine to form methionine. When dimethylaminoethanol was omitted from the diets, the chicks grew comparatively slowly and showed a high incidence of perosis unless choline was added.

In a second series of experiments a diet was used containing dried peas as the main source of protein. This was lower in methionine than the purified diet which contained soybean protein. It was also deficient in vitamin B₁₂ and further studies of the relationship between this vitamin and homocystine were thus made possible.

The addition of aureomycin was found in preliminary experiments to improve growth without affecting the qualitative responses to vitamin B₁₂, methionine or choline, and it was hence included in all the basal diets at a level of 20 mg/kg.

EXPERIMENTAL

Chicks were obtained from a breeding flock (Barred Plymouth Rock × New Hampshire) which was maintained on a corn-soybean basal diet, deficient in vitamin B₁₂, as described previously (Stokstad et al., '49). The chicks at hatching were segregated in groups of 10 to 12, each group with the same

average weight of 42 to 45 gm, and were kept in heated battery brooders in an air-conditioned room at 28°C. The diets were mixed at frequent intervals and the main supply was kept in a refrigerator. Several kilograms of DL-homocystine were kindly prepared by Dr. K. A. Burke using a method which was essentially that of Butz and DuVigneaud ('32) with a few modifications as follows: 500 gm of methionine were dissolved in 2 l of 18N H₂SO₄. This solution was refluxed slowly for 8 hours. After cooling to room temperature, the solution was neutralized with 15N NH₄OH until slightly acid to litmus, adding the ammonium hydroxide dropwise with rapid stirring and keeping the solution cool. The precipitate of homocystine was filtered and washed with cold water. The wet cake was then redissolved in a minimal amount of 2.5N HCl (about 1 l). This solution can be decolorized with charcoal if necessary and the homocystine reprecipitated by the addition of NH₄OH. The precipitate was again filtered and washed thoroughly with cold water. This preparation decomposed at about 265° to 268°C. and on microbiological assay showed the presence of less than 1% methionine.

Diet 1 consisted of glucose (Cerelese) 61.5 gm, soybean protein¹ 25 gm, calcium gluconate 5 gm, corn oil *plus* vitamins A, D and E (Stokstad et al., '49) 3 gm, bone ash 2 gm, salt mixture (Stokstad et al., '49) 2 gm, L-cystine 0.3 gm, dimethylaminoethanol HCl 0.2 gm, inositol 0.1 gm, aureomycin HCl 2 mg, and the following vitamin mixture: niacinamide 5 mg, calcium pantothenate 5 mg, thiamine HCl 1 mg, riboflavin 1 mg, pyridoxine HCl 1 mg, vitamin K compound (1-acetoxy-2-methyl-4-naphthyl sodium phosphate) 0.5 mg, and biotin 0.02 mg. The soybean protein was assayed for methionine content as follows²: 10 gm of protein were hydrolyzed by autoclaving for 16 hours in 6N hydrochloric acid and the hydrolysate was concentrated to a tar, taken up with water and treated with 1 gm of charcoal. The filtrate from this procedure was assayed for its methionine content with *S. faecalis* and the protein was thus found to contain 1.5% of methionine.

¹ Drackett Co. "Industrial Protein 220," Cincinnati, Ohio.

² Kindly carried out by Dr. H. P. Broquist.

This indicated that diet 1 contained about 0.38% of methionine. Diet 2 was identical with diet 1 except that dimethylaminoethanol was omitted and 0.5 mg of pteroylglutamic acid was added. Diet 3 consisted of ground dried split green peas 70 gm, glucose 14 gm, gelatin 8 gm, bone ash 3 gm, corn oil plus vitamins A, D and E 1 gm, sodium chloride 0.5 gm, DL-tryptophan 0.2 gm, manganese sulfate 25 mg, aureomycin HCl 2 mg, and the same vitamin mixture as for diet 1 plus pteroylglutamic acid, 0.2 mg. The peas were found to contain 0.19% of methionine by microbiological assay and 22% protein (Kjeldahl). This indicated that about 0.20% methionine was present in diet 3, assuming that gelatin contains 0.8% methionine (Block and Bolling, '51). Two groups of chicks were simultaneously fed the same diet in each experiment and the weight data represent the average of two groups in the case of each value shown. The results of the first experiment are shown in table 1. Experiment 2 was a repetition of experiment 1 except that three of the diets containing betaine were omitted because betaine seemed no more effective than choline in producing growth in the presence of homocystine, and the results are shown in table 2.

The diets were planned so that the supplements were added singly and in various combinations, thus enabling their growth-promoting effect to be measured under a variety of conditions; for example, by subtracting the weight of 151 gm obtained in chicks with the diet supplemented with homocystine, choline and PGA from that of 324 gm obtained with homocystine, choline, PGA and vitamin B₁₂ (table 1) a figure of 173 gm is obtained as a measure of the response to vitamin B₁₂. In this manner the growth responses to homocystine, methionine, betaine, choline, vitamin B₁₂ and PGA were measured under a number of dietary conditions and the effect on growth of combinations of these various supplements was also observed.

The diet containing glucose, soybean protein and dimethylaminoethanol may be regarded as being deficient in methionine, "labile methyl," vitamin B₁₂ and folic acid. The deficiency

TABLE 1
Results of experiment 1 in which diet 1 was fed to duplicate groups of 12 chicks each together with various supplements

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CON- SUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF						PGA	
			Homo- cystine	Betaine + homo- cystine	Choline + homo- cystine	Meth- ionine	Betaine	Choline		B ₁₂
	gm	gm								
None	75 ± 4.2	9.2								
DL-homocystine (6 gm)	74 ± 5.6	10.3	—1							
Homocystine + betaine (2 gm)	119 ± 7.7	11.3	11	44 ¹	65 ¹		45 ¹	66 ¹		
Homocystine + choline (2 gm)	140 ± 7.3	14.4	—1							
DL-methionine (6 gm)	176 ± 8.3	15.3				101 ¹				
Methionine + choline	200 ± 6.9	17.5				59 ¹		24 ¹		
Betaine	108 ± 5.9	5.9					33 ¹			
Choline	141 ± 6.9	13.9						66 ¹		
PGA (5 mg)	92 ± 7.1	9.3								17
Homocystine + PGA	88 ± 6.1	9.9	—4							14
Homocystine + betaine + PGA	144 ± 9.2	14.4		52 ¹	59 ¹		56 ¹	63 ¹		25 ¹
Homocystine + choline + PGA	151 ± 12.0	14.2	—4							11
Methionine + PGA	202 ± 12.1	18.7				110 ¹				26
Choline + PGA	155 ± 10.8	14.7						63 ¹		14
B ₁₂ (50 µg)	182 ± 10.5	16.0								
Homocystine + B ₁₂	242 ± 15.0	20.7	60 ¹						107 ¹	
Homocystine + betaine + B ₁₂	296 ± 13.1	21.3							168 ¹	
Homocystine + choline + B ₁₂	315 ± 11.0	22.5	82 ¹	114 ¹	133 ¹		54 ¹	73 ¹	177 ¹	
Methionine + B ₁₂	312 ± 8.6	22.5								
Choline + B ₁₂	233 ± 17.0	21.2				130 ¹		51 ¹	136 ¹	
B ₁₂ + PGA	255 ± 12.7	20.1							92 ¹	73 ¹
Homocystine + B ₁₂ + PGA	321 ± 12.6	22.5	66 ¹						163 ¹	233 ¹
Homocystine + betaine + B ₁₂ + PGA	338 ± 12.1	22.5		83 ¹	69 ¹		17		233 ¹	79 ¹
Homocystine + choline + B ₁₂ + PGA	324 ± 12.8	22.5	69 ¹			94 ¹		3	194 ¹	42 ¹
Methionine + B ₁₂ + PGA	349 ± 12.9	22.6							173 ¹	9
Choline + B ₁₂ + PGA	255 ± 7.8	20.0						0	147 ¹	37 ¹
									100 ¹	22

¹ Significant at the 5% level.

TABLE 2
Results of experiment 2 in which diet 1 was used. Most of the supplements were identical with those in experiment 1

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF											
			Homo- cystine	Betaine + homo- cystine	Choline + homo- cystine	Meth- ionine	Betaine	Choline	B ₁₂	PGA				
None	65 ± 2.8	9.0												
DL-homocystine (6 gm)	68 ± 5.5	9.5	3											
Homocystine + betaine (2 gm)	124 ± 6.0	10.5	4	59 ¹	40 ¹			56 ¹		37 ¹				
Homocystine + choline (2 gm)	105 ± 4.6	9.9	—8											
DL-methionine (6 gm)	183 ± 9.7	16.3							118 ¹					
Methionine + choline (2 gm)	200 ± 10.9	17.1							87 ¹					
Betaine (2 gm)	120 ± 6.7	9.8												
Betaine (4 gm)	121 ± 4.8	9.8												
Choline (2 gm)	113 ± 6.4	9.6												
Choline (4 gm)	145 ± 8.9	11.1												
PGA (5 mg)	73 ± 4.8	9.3												8
Homocystine + PGA	69 ± 6.2	9.9	—4											1
Homocystine + choline (2 gm) + PGA	133 ± 7.9	15.6	—17		60 ¹									28 ¹
Methionine + PGA	162 ± 12.5	16.0							89 ¹					—21
Choline (2 gm) + PGA	150 ± 7.9	15.2												37 ¹
B ₁₂ (50 µg)	185 ± 9.5	15.2												
Homocystine + B ₁₂	222 ± 10.4	17.1	37 ¹											120 ¹
Homocystine + choline (2 gm) + B ₁₂	305 ± 10.2	19.5	74 ¹											154 ¹
Methionine + B ₁₂	279 ± 9.7	18.8												200 ¹
Choline (2 gm) + B ₁₂	231 ± 8.7	16.3							94 ¹					96 ¹
B ₁₂ + PGA	247 ± 9.0	19.0												118 ¹
Homocystine + B ₁₂ + PGA	282 ± 11.6	19.0	35 ¹											174 ¹
Homocystine + choline (2 gm) + B ₁₂ + PGA	311 ± 11.0	19.0	78 ¹			64 ¹								213 ¹
Methionine + B ₁₂ + PGA	317 ± 9.6	20.0												6
Choline (2 gm) + B ₁₂ + PGA	233 ± 11.2	16.6												178 ¹
														155 ¹
														38 ¹
														83 ¹
														2

¹ Significant at 5% level.

of methionine was not acute because the diet contained about 0.4% of methionine and 0.3% cystine was added. These figures may be compared with the level of 0.5% methionine in the presence of 0.4% cystine estimated by Almquist ('47) to be required for the growth of chicks.

On this basal diet the growth data obtained with various supplements indicated the following:

1. No growth response was obtained to homocystine when added singly to the basal diet in the absence of vitamin B₁₂.
2. In the presence of vitamin B₁₂, homocystine produced a growth response with or without the addition of folic acid.
3. No response was obtained to choline or betaine when vitamin B₁₂ and folic acid were both added. Perhaps this indicates that the presence of these two vitamins made other sources available, such as glycine and serine, of a "single-carbon fragment" for the methylation of dimethylaminoethanol so that dietary choline or betaine were not required.
4. In the absence of vitamin B₁₂ the response to a mixture of homocystine and choline or to a mixture of homocystine and betaine was equal only to the arithmetic sum of the individual responses to homocystine, choline or betaine *when fed separately*. However, with vitamin B₁₂ added the response to a mixture of homocystine and choline or to a mixture of homocystine and betaine was greater than the arithmetic sum of the responses to the components of the mixtures when fed individually. This may indicate that methylation of homocystine by choline or betaine proceeded only when vitamin B₁₂ was added to the diet.
5. The response to methionine was relatively unaffected by the presence or absence of vitamin B₁₂ or folic acid.
6. The response to vitamin B₁₂ was greater in the presence of folic acid than in its absence; and also was greater in the presence of homocystine than in its absence when methionine was omitted.
7. The response to folic acid was variable; it was greatest in the presence of vitamin B₁₂ with or without homocystine and it was diminished by adding choline. Perhaps this indi-

cates a role for folic acid in making glycine and serine available for the methylation of dimethylaminoethanol, for with preformed choline added to the diet the methylation of dimethylaminoethanol was no longer required to ensure the supply of choline for growth.

With dimethylaminoethanol omitted from the basal diet and with folic acid added, the deficiencies now become those of choline, "labile methyl," methionine, and vitamin B₁₂. This diet (diet 2) was used in experiments 3 and 4 and the results are shown in tables 3 and 4. In contrast to the results obtained on the diet with dimethylaminoethanol, responses to betaine in experiments 3 and 4 were markedly lower than the responses to choline. This failure to respond to betaine may be attributed to the dietary absence of the group $\text{CH}_3\text{NHCH}_2\text{CH}_2\text{OH}$ which the chick needs in the diet to prevent choline deficiency and which must be supplied in the form of either methylaminoethanol, dimethylaminoethanol, choline (Jukes et al., '45) ethyldimethylaminoethanol or diethylmethylaminoethanol, or be replaced by arsenocholine (Jukes and Welch, '42).

The growth responses to various supplements encountered in chicks on the purified diet with folic acid added and dimethylaminoethanol omitted were as follows:

1. Homocystine produced no response in the absence of vitamin B₁₂. It has been suggested elsewhere that dimethylaminoethanol might compete with homocystine for methyl groups (Gillis and Norris, '51). No evidence was seen for this in the present investigation, or homocystine would presumably have produced better growth responses in experiments 3 and 4 than in the corresponding groups in experiments 1 and 2.

2. The response to homocystine in the presence of vitamin B₁₂ was rather small and was consistently improved by choline or betaine.

3. Responses to choline were obtained under all conditions of supplementation but the responses were greatest in the presence of vitamin B₁₂. There was markedly less response

TABLE 4
Results of experiment 4, in which experiment 3 was repeated

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF									
			gm	gm	Homo- cystine	Betaine + homo- cystine	Choline + homo- cystine	Meth- ionine	Betaine	Choline	B ₁₂	
			gm	gm								
None	82 ± 4.2	8.6										
DL-homocystine (6 gm)	80 ± 5.9	8.0			— 2							
Homocystine + betaine (2 gm)	107 ± 5.1	9.4			— 4							
Homocystine + choline (2 gm)	120 ± 5.9	10.7			— 42 ¹							
DL-methionine (6 gm)	154 ± 9.6	14.3										
Methionine + choline	184 ± 11.4	17.6										
Methionine + betaine	159 ± 9.3	15.0										
Betaine (2 gm)	111 ± 4.9	9.3										
Betaine (4 gm)	120 ± 5.2	10.4										
Choline (2 gm)	162 ± 8.1	13.6										
Choline (4 gm)	142 ± 6.2	12.0										
B ₁₂ (50 µg)	154 ± 5.8	14.7										
Homocystine + B ₁₂	174 ± 7.0	15.0			20 ¹							
Homocystine + choline (2 gm)												
+ B ₁₂	306 ± 11.5	20.9			30 ¹			152 ¹			118 ¹	186 ¹
Homocystine + betaine (2 gm)												
+ B ₁₂	204 ± 7.4	15.8			42 ¹		50 ¹			30 ¹		97 ¹
Betaine (2 gm) + B ₁₂	162 ± 6.8	15.1										51 ¹
Methionine + B ₁₂	227 ± 5.0	18.5										73 ¹
Methionine + choline (2 gm)												
+ B ₁₂	302 ± 8.8	20.0									75 ¹	118 ¹
Methionine + betaine (2 gm)												
+ B ₁₂	224 ± 10.2	20.0										65 ¹
Choline (2 gm) + B ₁₂	276 ± 9.3	19.1								— 3	122 ¹	114 ¹

¹ Significant at 5% level.

to betaine than to choline and the greatest response to betaine was obtained on the unsupplemented basal diet.

4. The response to a mixture of homocystine and choline, or homocystine and betaine, was only equal to or was less than the arithmetic sum of the responses to homocystine, choline and betaine when fed separately, regardless of the presence or absence of vitamin B₁₂. This observation is difficult to interpret. Perhaps the growth response obtained to homocystine plus betaine in the presence of dimethylaminoethanol in experiments 1 and 2 was due to the formation of methionine and its subsequent utilization in the methylation of dimethylaminoethanol.

5. The responses to methionine were less than were encountered in experiments 1 and 2 and no response was obtained to methionine when B₁₂ and choline were both added, thus leading to the conclusion that the growth-promoting effect of methionine when both B₁₂ and choline were not present was partly due to the activity of methionine as a source of labile methyl groups which could also be supplied by choline.

6. The response to vitamin B₁₂ was greater in the presence of homocystine than in its absence and was greatest in the presence of choline. Evidently, choline and vitamin B₁₂ could not function interchangeably in promoting growth.

The incidence of perosis in the various groups in experiments 1 and 2 was low. However, in experiments 3 and 4 a high incidence of perosis was seen in certain groups and all the chicks were examined at 25 days of age and "scored" for perosis. During the examination, the individual who "scored" the chicks did not know which diets they received. The system of "scoring" which was employed consisted of assigning a value of 0, 10 or 20 to each hock joint of each bird depending on the absence of perosis (0 points), the presence of a mild but definite degree of perosis (10 points), or the presence of severe and deforming perosis (20 points). The maximum average score thus was 40 points and the results are summarized in table 5, in which it will be noted that excellent agreement was obtained between replicate treatments. Perosis

TABLE 5

[illegible]

was correlated with the combined presence of vitamin B₁₂ and absence of choline. Betaine appeared to aggravate perosis slightly in the absence of vitamin B₁₂.

In the next series of experiments, diet 3 was used, which is closely similar to a diet used in a previous investigation for the simultaneous production of deficiencies of methionine and vitamin B₁₂ (Jukes and Stokstad, '51a). Excellent agreement was obtained in the results of the two experiments on diet 3, as may be seen from tables 6 and 7. It is evident that diet 3 was markedly deficient in methionine and it was also deficient in vitamin B₁₂ and partially deficient in "labile methyl." The response encountered on diet 3 were as follows:

1. Little or no response was obtained to homocystine in the absence of added B₁₂ unless betaine or choline was added, in which case a good response was obtained to homocystine.

2. There was a marked response to homocystine in the presence of vitamin B₁₂ which was improved only slightly by choline or betaine. Apparently the labile methyl donors present in the basal diet (perhaps in the peas) were used effectively only when vitamin B₁₂ was added.

3. Regardless of the presence or absence of vitamin B₁₂, the response to choline or betaine was quite small unless homocystine was added, leading to the conclusion that choline and betaine were functioning chiefly for the methylation of homocystine.

4. The responses to mixtures of homocystine and choline, or homocystine and betaine, were markedly greater than the arithmetic sum of the responses to these substances when fed alone in the absence of vitamin B₁₂. This augmenting effect of choline or betaine on the response to homocystine was somewhat less in the presence of vitamin B₁₂ than in its absence.

5. The response to methionine was remarkably constant regardless of the presence of betaine or choline added separately or in addition to vitamin B₁₂.

TABLE 6
Growth of chicks in experiment 5 on diet 3 (deficient in methionine and containing added PGA) with various supplements

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF					
			Homo- cystine	Betaine + homo- cystine	Choline + homo- cystine	Meth- ionine	Betaine	Choline
	gm	gm						
None	69 ± 2.0	9.2						
D,L-homocystine (6 gm)	82 ± 3.1	10.1	13 ¹					
Homocystine + betaine (2 gm)	142 ± 13.0	14.7	60 ¹				60 ¹	
Homocystine + choline (2 gm)	165 ± 7.8	14.9	87 ¹		96 ¹	169 ¹		83 ¹
D,L-methionine (6 gm)	238 ± 13.1	18.8				180 ¹	24	
Methionine + betaine	262 ± 11.1	19.4				169 ¹		9
Methionine + choline	247 ± 11.3	18.8					13 ¹	
Betaine	82 ± 2.4	10.0						9 ¹
Choline	78 ± 2.7	9.4						
B ₁₂ (50 µg)	103 ± 3.2	10.9						34 ¹
Homocystine + B ₁₂	270 ± 16.7	20.5	167 ¹					188 ¹
Homocystine + betaine + B ₁₂	330 ± 14.1	20.8	217 ¹	227 ¹			60 ¹	188 ¹
Homocystine + choline + B ₁₂	293 ± 16.4	20.3	181 ¹		190 ¹			128 ¹
Methionine + B ₁₂	304 ± 13.0	20.0				201 ¹		66 ¹
Betaine + B ₁₂	113 ± 4.0	12.1					10 ¹	31 ¹
Choline + B ₁₂	112 ± 5.2	13.4						34 ¹
Methionine + betaine + B ₁₂	291 ± 10.3	21.6				178 ¹	—13	29
Methionine + choline + B ₁₂	327 ± 13.5	21.6				215 ¹		34 ¹
Methionine (2 gm) + B ₁₂	274 ± 8.2	22.5				171 ¹		80 ¹
Methionine (4 gm) + B ₁₂	311 ± 10.5	22.5				208 ¹		

¹ Significant at 5% level.

TABLE 7
Growth of chicks in experiment 6, a repetition of experiment 5

ADDITION PER KILOGRAM OF BASAL DIET	25-DAY WEIGHT	FEED CONSUMED PER DAY	INCREASE IN AVERAGE WEIGHT (IN GM) FOLLOWING ADDITION OF				
			Homo- cystine	Betaine + homo- cystine	Choline + homo- cystine	Meth- ionine	B ₁₂
None	74 ± 2.3	9.1					
DL-homocystine (6 gm)	85 ± 4.9	9.1	11				
DL-homocystine + betaine (2 gm)	151 ± 7.9	13.5	67 ¹	77 ¹	89 ¹		78 ¹
DL-homocystine + choline (2 gm)	163 ± 6.8	14.4	77 ¹				
DL-methionine (6 gm)	217 ± 7.5	17.8				143 ¹	
Methionine + betaine	242 ± 10.4	18.1				158 ¹	19
Methionine + choline	236 ± 13.8	18.3				150 ¹	10 ¹
Betaine	84 ± 3.3	9.2					12 ¹
Choline	86 ± 1.9	9.6					
B ₁₂ (50 µg)	108 ± 5.7	14.5					34 ¹
Homocystine + B ₁₂	254 ± 11.0	17.3	146 ¹				169 ¹
Homocystine + betaine + B ₁₂	315 ± 14.8	21.3	200 ¹	207 ¹			164 ¹
Homocystine + choline + B ₁₂	330 ± 12.2	20.8	218 ¹		222 ¹		167 ¹
Homocystine + B ₁₂	284 ± 12.3	20.8				176 ¹	67 ¹
Methionine + B ₁₂	115 ± 5.0	13.3					31 ¹
Betaine + B ₁₂	112 ± 4.7	13.9				7	26 ¹
Choline + B ₁₂	292 ± 11.7	21.0				177 ¹	50 ¹
Methionine + betaine + B ₁₂	302 ± 15.9	20.8				190 ¹	66 ¹
Methionine + choline + B ₁₂						—7	18

¹ Significant at 5% level.

6. The response to vitamin B₁₂ was very marked in the presence of homocystine with or without choline or betaine; it was moderate in the presence of methionine, and small on the basal diet with or without betaine or choline.

The effect of methionine and homocystine in the presence and absence of vitamin B₁₂ on the growth of chicks is shown in figure 1, which strikingly illustrates the relation of vitamin

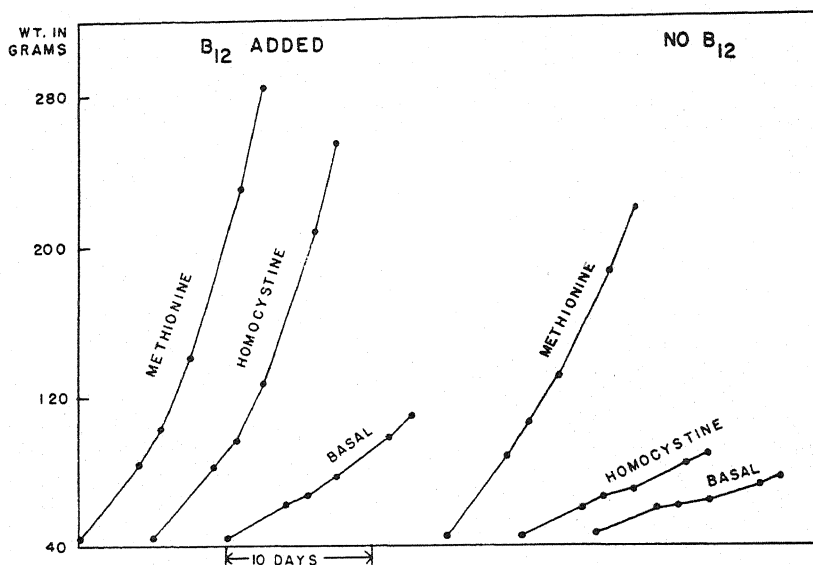


Fig. 1. Growth responses of chicks on diet 3 to methionine and homocystine, with and without vitamin B₁₂. Basal diet 3 was used (see table 7).

B₁₂ to the utilization of homocystine in chicks. Evidently a factor was present in the basal diet which enabled homocystine to replace methionine for growth in the presence of vitamin B₁₂. It was thought possible that the factor in the basal diet might be choline, since Engel ('43) has reported that dried peas contain 0.29% of this substance. Evidently the choline furnished by the basal diet was utilized far less efficiently for the methylation of homocystine in the absence than in the presence of supplementary vitamin B₁₂ as shown by the

following data which are average weights taken from tables 6 and 7.

ADDITION TO BASAL DIET	AVERAGE WEIGHT AT 25 DAYS		INCREASE DUE TO HOMOCYSTINE
	Without homocystine	With homocystine	
	<i>gm</i>	<i>gm</i>	
None	72	84	12
Vitamin B ₁₂	106	262	156

In contrast, *added* choline seemed to be utilized to a marked extent for methylation even in the absence of vitamin B₁₂ as judged by the finding that chicks on the diet supplemented with homocystine *plus* choline weighed twice as much (164 gm) as the chicks on the diet supplemented with choline alone (82 gm). When calculated similarly, the increase due to methionine was 156 gm without vitamin B₁₂ and 188 gm with vitamin B₁₂ in the diet, showing that the utilization of methionine was relatively unaffected by B₁₂.

Another possible explanation for the growth-promoting effect of homocystine when added to methionine-deficient diets arises from observations which have been made with rats indicating that in the presence of ample vitamin B₁₂ and PGA, methylation of homocystine may take place without the addition of a dietary supply of "methyl donors" such as choline or betaine (Bennett, '50). Glycine and serine, by giving rise to formate (Sakami, '49; Siekevitz and Greenberg, '50; Shemin, '46) which could serve in turn as a precursor of the methyl group of methionine in experiments with the tissues of rats and guinea pigs (Siekevitz and Greenberg, '50; Shemin, '46; Welch and Sakami, '50) are obvious possibilities as sources of the "single-carbon" fragment which can form a methyl group. An experiment was carried out to investigate this point by extracting the ground peas with hot alcohol to remove choline and possibly betaine. Such a procedure should not affect the glycine and serine in the diet which are derived both from pea protein and gelatin. The results of the experiment are shown in table 8. It may be seen that alcohol-extraction largely removed the component of the basal diet which

was presumably responsible for the methylation of homocystine in the presence of vitamin B₁₂ and PGA. Only a minor role can therefore be assigned to glycine and serine in the transformation of homocystine to methionine under the conditions of these experiments and the effectiveness of choline as a "methyl donor" is emphasized.

TABLE 8

Effect of various supplements on growth of chicks on diet 3 which was modified by extracting the ground peas with hot ethanol

ADDITION PER KILOGRAM OF BASAL DIET	AVE. WEIGHT OF CHICKS AT 25 DAYS (GM)	
	Without supplemental choline	2 gm choline added per kilogram of diet
None	68	82
DL-homocystine, 6 gm	69	144
Vitamin B ₁₂ , 50 µg	90	112
Homocystine + B ₁₂	108	295

DISCUSSION

The ability of the chick to form methionine from choline and homocystine was first indicated by the experiments of Klose and Almquist ('41). Their chicks were placed on a "standard chick mash" for a week preceding the experiments, and this procedure makes it probable that vitamin B₁₂ deficiency was not encountered.

It was found in our laboratory (Jukes et al., '50) that vitamin B₁₂-deficient chicks on a purified diet containing soybean protein would not show a growth response to either homocystine or homocystine *plus* betaine. In the present investigation vitamin B₁₂-deficient chicks on a similar diet did not respond to homocystine but responded to homocystine *plus* betaine (tables 1 to 4). The reason for this apparent disparity could well be that the chicks in the earlier investigation may have been more completely depleted of vitamin B₁₂, which is evidently needed for the methylation of homocystine by the chick. Variations in the extent of B₁₂ deficiency will therefore influence the response of chicks to homocystine

and betaine, thus accounting for differences in various investigations (Jukes et al., '50; Gillis and Norris, '51).

Folic-acid deficiency in chicks has been shown to depress the formation of methionine from homocystine by liver homogenates *plus* choline or betaine (Dinning et al., '51). Similarly in rats, liver homogenates from vitamin B₁₂-deficient animals showed a lower ability to form methionine from homocystine *plus* choline or betaine as compared with homogenates from rats receiving vitamin B₁₂. The close interrelationship of folic acid and vitamin B₁₂ in this and other biochemical processes affords an interesting sidelight to the circumstances surrounding the numerous observations, which are reviewed elsewhere (Jukes and Stokstad, '51b), that either folic acid or vitamin B₁₂ will produce a hemopoietic response in pernicious anemia.

In the present investigation the response to pteroylglutamic acid was lowered when B₁₂ and choline were both present in the diet (tables 1 and 2), perhaps indicating that these substances in combination "spare" the folic acid requirement of chicks.

SUMMARY

1. A study was made of the responses of vitamin B₁₂-deficient chicks to homocystine, betaine, choline, methionine, vitamin B₁₂ and folic acid as measured by early growth on diets which were deficient in several respects. A "factorial" design of adding the supplements enabled their interrelationships to be measured.

2. The response to homocystine was markedly increased by vitamin B₁₂ under a variety of dietary conditions. However, in the absence of vitamin B₁₂ homocystine often actually depressed growth.

3. The response to homocystine in the presence of added vitamin B₁₂ was increased by the addition of choline or betaine. However, when vitamin B₁₂ was not added, the response to homocystine plus choline or betaine added as a mixture was no greater than the sum of the responses to homocystine and choline or betaine when tested individually.

4. Choline appeared to be highly effective as a "methylating" agent for homocystine in the presence of vitamin B₁₂ on a diet which was markedly deficient in methionine. There was no indication that the amino acids in the basal diet could effectively replace choline for this purpose.

5. The incidence of perosis on a purified diet without added dimethylaminoethanol was high when vitamin B₁₂ was added and choline was omitted. The incidence was not reduced by adding betaine.

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NUTRITIONAL EVALUATION OF FOOD PROTEINS BY MEASURING AVAILABILITY OF AMINO ACIDS TO MICROORGANISMS

I. COTTONSEED PROTEINS ¹

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To evaluate the changes which take place in a protein when a food is processed, the essential amino acid content and the change in digestibility of the protein before and after processing should be known. Many workers have shown that heat changes the nutritive value of a protein (McCollum and Davis, '15; Block et al., '34; Eldred and Rodney, '46; Patton and Hill, '48; Riesen et al., '47; Evans and Butts, '48; Pader et al., '48; Stevens and McGinnis, '47; Mader et al., '49; Olcott and Fontaine, '41; Ingram et al., '50; Dechary and Altschul, '49; Simon and Melnick, '50; Evans and Butts, '49; Hou et al., '49; Baldwin et al., '51; Hadden et al., '50). Two types of heat inactivation apparently take place; one the destruction of amino acids, the other a binding of the amino acids so that they are not liberated by digestion *in vivo* or by enzymatic hydrolysis *in vitro*, but are liberated by acid hydrolysis (Evans and Butts, '48). It is the purpose of this investigation to study these two types of inactivation on a series of foods, processed under known conditions, and to attempt to evaluate the nutritional quality of the various food proteins from the data obtained.

¹ This is the first of a series of papers on the availability of amino acids in raw and processed foods.

Considerable time was expended to develop an enzyme system by which the extent of hydrolysis could be made reproducible. It was found that by rigid control of conditions good reproducibility could be obtained.

This paper deals with results obtained on a series of cottonseed meals prepared under known conditions. Rat-feeding tests to evaluate the protein quality were carried out at the same time as microbiological assays of acid and enzyme digests, and a comparison was made of the two methods of studying protein damage. The results obtained on these meals by microbiological assay of enzyme digests agree quite well with results obtained from animal-feeding experiments using the same meals.

EXPERIMENTAL

As part of the research program inaugurated by the Southern Regional Research Laboratory, in cooperation with the cottonseed industry, a series of 10 cottonseed meals (series 1) was received which were prepared under different processing conditions, particularly the temperature and time of cooking (Hadden et al., '50; table 1). The meal used as a standard on the first series was an essentially gland-free meal (Vix et al., '49), and was prepared at the Southern Regional Research Laboratory. Cooking is not employed in making gland-free meals. Numbers 1 to 9 were prepared by the South Texas Cotton Oil Company with the cooperation of the Southern Regional Research Laboratory. Numbers 2 and 9 are hydraulic-press meals and the remaining 7 are screw-press (Hadden et al., '50). Processing conditions were established and chemical properties determined at the Southern Regional Research Laboratory and are given in table 1.

Series 5 consisting of 15 experimental meals was prepared, taking advantage of the information obtained from series 1. Five of these were analyzed in the same manner as was done with series 1. The processing data for these meals are also given in table 1. The standard in this series was an unheated cottonseed meal which had been treated with hexane and ethyl methyl ketone.

TABLE 1
Processing conditions and chemical properties of cottonseed meals

MEAL NO.	MAXIMUM COOKING TIME	MAXIMUM TEMPERATURE	LIPIDS	TOTAL GOSSYPOL	FREE GOSSYPOL-LIKE MATERIAL	MOISTURE	TOTAL ³ NITROGEN	SOLUBLE NITROGEN
	min.	°F.	%	%	%	%	%	% of total
Standard ¹	less than 1%	Series 1	0.080	8.0	12.0	83.3
1	20	234	3.5	0.63	0.010	4.6	7.56	11.7
3	20	261	3.6	0.50	0.020	4.4	7.69	10.0
4	40	262	3.8	0.51	0.020	4.5	7.73	10.3
5	100	279	5.7	0.14	0.030	3.7	7.04	8.2
6	20	200	3.4	0.43	0.010	4.1	7.10	15.9
7	70	200	3.4	0.43	0.010	4.7	7.25	12.8
8	70	180	3.3	0.40	0.010	4.6	7.46	14.3
2	36	230	5.6	0.70	0.108	7.6	7.40	39.2
9	76	240	5.6	0.41	0.047	6.6	7.66	20.8
Standard ²	0.2	Series 5	0.012	10.25	10.38	70.7
1	37	230	3.7	0.115	0.028	6.54	7.80	15.3
9	26	180	6.4	0.658	0.019	6.37	7.52	31.7
10	26	180	6.3	0.566	0.044	6.26	7.25	41.4
13	26	200	5.9	0.613	0.028	5.90	7.04	26.5

¹ Essentially gland-free meal, hexane extracted.

² Extracted with methyl-ethyl ketone and hexane.

³ Moisture and ash free.

The effect of different processing conditions on the protein quality of the meals was evaluated by feeding them at a level of 10% protein ($N \times 5.3$) to weanling male rats from the stock colony of this laboratory.² The other ingredients of the ration consisted of salt mixture (Jones and Foster, '42) 4%, corn oil³ 3%, lard 12%, vitamin A and D concentrate⁴ 0.05%, inositol 0.1%, choline chloride 0.2% and sucrose to make 100%. In addition, the following vitamins were added per kilogram ration: thiamine hydrochloride, 5 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 5 mg; nicotinic acid, 5 mg; calcium *d*-pantothenate, 25 mg; *p*-aminobenzoic acid, 300 mg; α -tocopherol acetate, 25 mg; 2-methyl 1,4-napthoquinone, 2 mg; biotin, 100 μ g; and folic acid, 2 mg. Some of the animals were fed vitamin B₁₂⁵ at a level of 30 or 50 μ g/kg. In feeding the meals of series 5, the corn oil was decreased to 2%, the lard to 8% and the sucrose increased correspondingly. Food and water were fed ad libitum, and the weight changes and food intakes were determined weekly. Ten animals were fed each diet, and litter mates were distributed among the various groups in a random fashion. The feeding trials were continued for 6 weeks, and the protein efficiencies (grams gain per gram of protein consumed) of the meals determined.

An acid hydrolysis was made on each sample and the essential amino acids were determined microbiologically using the methods developed in this laboratory (Horn, Jones and Blum, '50). Little information suggesting differences in meals was obtained by assay of the acid hydrolysates. However, vast differences in the nutritive value of these meals were obtained from the animal-feeding experiments. It was believed, therefore, that analysis of an enzyme digest might show why the differences occurred. Since the amount of growth is limited in the microbiological method by the availa-

² The strain of rats is the result of mating an albino with a black and white hooded strain. The litters may include white, black and black and white rats.

³ Mazola.

⁴ Squibb's Navitol containing 65,000 USP units of vitamin A and 13,000 USP units of vitamin D per gram.

⁵ Squibb's Rubramin or Merck's Cobione.

ble amino acids, the amino acids in the hydrolysates analyzed must be free or in the form of small peptides. Any processing tending to inhibit the digestion of the protein so that the amino acids are no longer freed should then be detected by the microbiological method.

An enzyme system was developed using pepsin, trypsin and hog mucosa for successive 24-hour periods. After this 72-hour digestion period the essential amino acids were determined microbiologically on the solutions. That reproducible results were obtained by this method was shown by the fact that the apparent amino acid availabilities of replicate samples agreed within $\pm 8\%$.

In our earlier work the assay levels of the enzyme digests did not check, varying as much as 50%. Investigation into the matter showed that the buffer in the digests was causing a constant increase in titration for all points on the curve. This was true for all the amino acid standard curves and indicated that the "blank" contained negligible amounts of the amino acid being assayed. When corrections for the titration attributable to the blank were made, the assay levels of enzyme digests agreed well within the experimental error of microbiological assays.

RESULTS

The results of the rat-feeding trials are given in table 2. The results from the microbiological assays of both acid and enzyme hydrolysates are given in table 3. In order to compare the various meals the results from the microbiological assays were calculated to 16% nitrogen and expressed as per cent of the standard. The assays on the acid hydrolysates (table 3) show that except for number 5 there is very little destruction of amino acids by the processing. On number 5, some of the lysine, methionine and histidine appeared to be destroyed by the processing.

Examination of the results on enzyme hydrolysates (table 3) shows that there are differences in the availability of the amino acids in the various meals. Assuming the amount

of the amino acids obtained on the standard (unheated) meal to be the maximum amount freed by the enzyme system used, the heated meals then show differences in digestibility due to the processing. The heat and other processing conditions have caused a change in the protein of the cottonseed so that the enzymes cannot digest it with the same facility as they do the standard meal. All the amino acids appear to be affected. Some amino acids are affected more than others,

TABLE 2
Protein efficiencies of various cottonseed meals

SERIES 1		SERIES 5 ³	
Meal no.	Protein efficiency	Meal no.	Protein efficiency
1	1.83	1	2.02
2	2.01	9	2.16
3	1.73	10	2.46
4	1.70	10 (no B ₁₂)	2.28
5	0.52	13	2.31
6	2.26	Standard ⁴	2.87
7	2.01		
8	2.16		
9	1.69		
Standard ¹	2.50		
Standard + B ₁₂ ²	2.70		

¹ Hexane extracted.

² Fifty micrograms per kilogram.

³ Thirty micrograms B₁₂ added per kilogram of ration except where indicated.

⁴ Ethyl-methyl ketone and hexane extracted.

depending on the variables of processing. For example, number 4 has less arginine and isoleucine available but much more histidine, lysine and threonine than number 2.

Differences in availability of the various amino acids in the enzyme digests of series 1 may be illustrated as follows: the amounts of amino acids liberated from the standard meal by the enzyme system used when compared with the amounts liberated by the acid hydrolysis of the standard show arginine, lysine and histidine to be 79%, 52% and 33% available. This is the maximum per cent available by this system. Any

TABLE 3

Effect of processing on content and availability of essential amino acids of cottonseed meals¹

	ARGININE		HISTIDINE		ISOLEUCINE		LEUCINE		LYSINE	
	A	E	A	E	A	E	A	E	A	E
Std.	100	100	100	100	100	100	100	100	100	100
	(12.37) ²	(9.77)	(2.86)	(0.93)	(3.53)	(2.17)	(6.08)	(3.85)	(4.93)	(2.54)
1	96.4	83.8	96.2	79.6	92.6	98.6	99.0	94.3	100	48.0
2	97.0	93.6	92.7	53.7	103.1	97.2	103.1	87.0	96.6	43.3
3	94.9	84.8	95.1	58.1	98.0	74.6	95.0	76.6	95.3	67.7
4	96.0	80.4	90.9	69.9	102.0	76.9	100.0	79.2	96.4	65.7
5	94.6	69.1	87.9	40.8	94.0	86.1	94.0	77.1	80.7	31.9
6	100.6	88.0	102.8	77.4	99.7	100.0	95.2	101.3	103.4	71.7
7	100.6	88.1	90.9	88.2	103.1	100.0	106.1	89.3	99.4	77.2
8	97.8	88.1	102.8	77.4	100.9	100.5	96.8	99.2	98.2	74.0
9	99.0	89.2	93.7	95.4	101.9	77.9	96.7	102.3	97.4	34.6
Std.	93.1	86.6	96.2	95.4	106.5	96.7	100.0	94.5	96.1	93.7
1	96.0	73.5	86.0	64.5	98.3	97.7	98.7	91.2	92.9	59.8
9	99.8	70.2	85.3	83.9	107.3	91.2	97.5	100.5	98.4	58.3
10	95.1	80.1	90.5	84.8	108.2	103.2	99.6	91.4	97.4	60.2
13	100.3	74.8	91.6	81.7	115.0	100.0	100.6	94.6	89.0	59.8
Comm'l.	92.3	70.8	86.1	65.6	104.0	97.2	96.7	82.1	89.9	62.6
Av.	96.9		93.2		102.3		98.6		95.4	

	METHIONINE		PHENYLALANINE		THREONINE		TRYPTOPHAN		VALINE	
	A	E	A	E	A	E	A	E	A	E
Std.	100	100	100	100	100	100	100	100	100	100
	(1.50)	(1.12)	(5.41)	(4.31)	(3.69)	(2.48)	(0.78)	(4.98)	(2.90)	
1	92.0	50.9	93.9	79.8	107.7	60.1	79.5	102.0	77.6	
2	86.7	44.6	102.0	83.8	114.1	57.7	71.8	104.2	70.0	
3	93.3	50.0	92.2	81.4	99.5	60.1	80.8	101.6	73.8	
4	90.0	51.8	94.8	76.9	102.4	82.3	85.9	102.0	79.0	
5	81.3	35.7	88.4	81.2	107.3	46.8	50.0	101.6	71.0	
6	102.0	49.1	90.2	89.3	105.2	64.9	80.8	103.0	92.8	
7	94.0	57.1	94.6	86.1	96.5	60.9	76.9	102.0	101.4	
8	113.3	59.8	91.5	86.5	103.5	100.0	80.8	100.0	103.5	
9	104.0	44.6	90.4	76.8	95.7	81.1	66.7	101.6	94.8	
Std.	104.0	76.8	97.6	103.3	101.4	78.6	99.7	107.2	96.9	
1	82.0	42.9	93.2	77.3	96.8	79.4	62.8	104.4	102.1	
9	86.7	46.4	96.5	80.3	103.0	90.3	70.5	106.4	100.3	
10	82.0	50.9	94.3	84.9	113.3	98.0	70.5	110.4	113.8	
13	90.7	46.4	93.2	83.5	117.3	80.7	68.0	102.4	101.7	
Comm'l.	82.0	48.2	97.0	86.8	104.6	64.1	69.2	100.0	91.4	
Av.	92.9		94.0		104.5			103.3		

¹ Expressed as per cent of unprocessed meal.² Actual amino acid content of standard calculated to 16% nitrogen.

deviations from these availabilities reflect changes due to processing. In series 5 it can be seen that, with the exception of the commercial meal, histidine, lysine, arginine, tryptophan, and methionine were less available in the heated than in the unheated meal; leucine, isoleucine, and valine were as available as in the standard.

Correlation of microbiological and animal feeding results

In order to evaluate the results obtained from the amino acid assays on both series, a "Nutritive Index" was devised as follows: the amount of the 10 amino acids set free by the enzyme system for the standard of series 1 was taken as 100%. The values obtained for the other meals were divided by the values obtained for the standard of series 1. This was done for each essential amino acid, giving a series of percentages for each meal. These, when added together and divided by 10, gave the Nutritive Index for that meal or average availability to the microorganisms of the 10 essential amino acids. For example, the enzyme digest of the standard for series 1 when assayed for lysine gave 2.54%; the assay for meal number 1 gave 1.22%; therefore the fraction for lysine for meal number 1 was 0.48, or 48%. When this was done for all 10 essential amino acids for meal number 1, and the percentages added and divided by 10, the Nutritive Index for that meal was found to be 75.2% compared with 100% for the standard. The Index values can be obtained directly from table 3.

In the rat-feeding trials of series 1 vitamin B₁₂ was included in the ration in only one test. In series 5, vitamin B₁₂ was added in the testing of each meal, but one meal was also fed without B₁₂. In series 1 the values for protein efficiencies for the meal fed with and without B₁₂ were 2.70 and 2.50. In series 5 the values were 2.46 and 2.28. These values represent an increase of 8.00 and 7.9% in the protein efficiency due to the presence of the vitamin. In the microbiological assays the Nutritive Indices of both series are calculated

with the standard of series 1 as 100. In order to compare the rat-growth studies from the two series we have decreased the values for series 5 by 8%, the amount by which the nutritive value was increased by the presence of B₁₂. In order to compare the results of the rat-feeding trials and the amino acid assays, the results of the rat-feeding trials are expressed as per cent of the protein efficiency of the standard meal of series 1.

TABLE 4

Nutritive indices calculated from microbiological data compared with indices from rat-growth studies

NUMBER COTTONSEED MEAL	NUTRITIVE INDICES	
	From microbiological studies	From rat growth studies
Series 1		
Standard	100	100
8	87	86
7	83	80
6	82	90
9	76	68
1	75	73
4	75	68
3	71	69
2	70	80
5	59	22
Series 5		
Standard	92	106
10	84	90
9	80	80
13	79	85
1	75	74

In table 4 the Nutritive Indices obtained from the microbiological assays are compared with the Nutritive Indices obtained by rat feeding. In considering the relative order of nutritive value there is good correlation between the Indices.

It is believed that with a little more experience and refinement of the technique the enzyme microbiological method will prove to be a useful tool in evaluating protein foods with fair reliability.

It might be pointed out that attempts to correlate the feeding and microbiological methods by using one, two or even three of the most labile amino acids met with no success. It appears that consideration of all the essential amino acids must be given to evaluate the meal.

The results on chick feeding obtained by Milligan and Bird ('51) on the meals of series 1 agree favorably with our results.

SUMMARY

It has been demonstrated that various methods of processing cottonseed meals alter the nutritional value of the protein.

Two methods of evaluating these meals were used; the rat assay method, which showed that the nutritional value of these meals for the rat was considerably altered by the processing, and the microbiological method, which showed that the changes in nutritional value were due to a change in the availability of the amino acids. Different methods of processing affected the availability of different amino acids. The assay of acid hydrolysates showed that only very severe temperatures and pressures caused any destruction of the amino acids. Comparison of results from assays of acid and enzyme hydrolysates by the microbiological method showed that, although the amino acids were present, some of them were bound in such a way that they were no longer available to the microorganism.

An enzyme digestion system has been worked out which appears to give good reproducibility of results; the availability of the digestion products to the microorganisms correlated well with the results of rat feeding.

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THE EFFECT OF DIETARY FAT AND CALORIC RESTRICTION ON PROTEIN UTILIZATION ¹

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FOUR FIGURES

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The experiments of Willman et al. ('47) and Swanson ('51) indicate that fats have a greater nitrogen sparing effect than carbohydrates in rats fed a low caloric diet. Similar results have been reported in man by Schwimmer and McGavack ('48). On the other hand, Allison et al. ('46) reported data on the dog that suggested little if any difference in the protein sparing action of fat or carbohydrate.

The present experiments were undertaken to investigate more fully the role of dietary fat with concomitant caloric restriction on the utilization of dietary protein in the dog. Since the incorporation of large amounts of fat into the diet might cause intestinal disturbances, digestibility studies were completed in normal and protein-depleted dogs.

METHODS

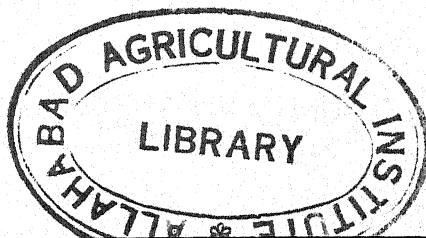
Adult dogs were fed the purified diet used in previous studies by Allison and Anderson ('45) as modified by Rosen-

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The data reported in this paper were submitted to the Graduate Faculty, Rutgers University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.



thal and Allison ('51). Since the caloric requirement of dogs varies somewhat with the size and breed, the results are expressed in terms of the average per cent of the calories required to maintain the body weight of adult dogs, as determined in this laboratory. Protein (Labco Casein) was incorporated in the diet and the animals were fed on the basis of grams protein nitrogen/day/kilogram probable body weight (Cowgill, '28). The caloric, fat (lard) and protein content of the diet was increased or decreased by substitution of carbohydrate.

Nitrogen balance indexes were determined by methods previously developed in this laboratory (Allison and Anderson, '45). The nitrogen balance index (K) is a function of the amount of nitrogen retained in the body of the animal and is defined as the rate of change of nitrogen balance with respect to absorbed nitrogen. The slope of the line then becomes the nitrogen balance index. This equation is constant in the region of negative or low positive nitrogen balance, but it becomes a decreasing variable in the region of high positive nitrogen balance. To determine the index, the dogs were placed on a protein-free diet for 7 days, followed by 8 days of protein feeding (two periods), and again followed by 5 days of protein-free feeding. Appropriate collections of urine and feces (with carmine markers) were made and analyzed for nitrogen by the Pregl micro-Kjeldahl method.

The digestibility of lard was determined by placing dogs on the diet containing egg albumin as the protein source. They received 0.12 gm nitrogen per day per kilogram of body weight and 100% of their caloric requirement. The fat in the food and feces was determined as the weight of free fatty acids by a modification of the wet extraction method described by Fowweather ('26). Plasma proteins were determined by the salt fractionation method of Howe ('21). After the normal data were obtained, the dogs were depleted by feeding a protein-free diet for 30 days, and the experiments were repeated on the depleted animals.

RESULTS

Allison et al. ('46) have shown that the nitrogen balance index equation becomes curvilinear in the region of positive balance. They also demonstrated that dogs fed restricted diets could not maintain positive nitrogen balance. The data presented in figure 1 show the effect of dietary protein on

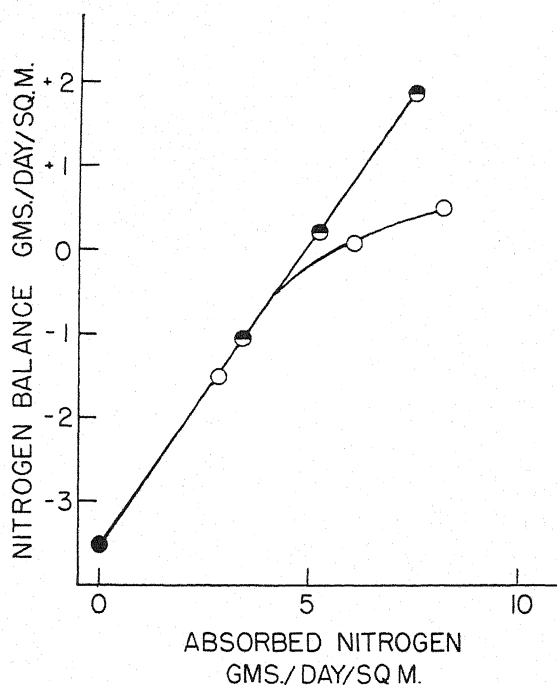


Fig. 1 Nitrogen balance versus absorbed nitrogen for dogs fed various protein intakes. The half black circles (●) represent dogs fed 100% of the caloric requirement and the open circles (○) represent dogs fed 25% of the caloric requirement. The solid circle (●) represents nitrogen excretion on a protein-free diet.

nitrogen balance at different caloric intakes. These data were obtained by using diets limited to a fat content of 5% of the calories. All of the experimentally determined values have been adjusted to the same NE_0 value in accordance with the equation $NB = K(AN) - NE_0$, where NB is nitrogen balance, K is the nitrogen balance index, AN is absorbed nitrogen and

TABLE 1
*The effect of dietary fat on the nitrogen balance index of casein obtained on dogs fed 0.15-0.20 gm casein
 nitrogen/day/kilogram body weight*

FAT CONTENT OF DIET	NO. OF DETER- MINATIONS	ABSORBED NITROGEN	NITROGEN BALANCE	PROTEIN- FREE NITROGEN BALANCE	DIGESTIBILITY ¹	NITROGEN BALANCE INDEX
% Cal.		gm/day/m ²	gm/day/m ² 100% Caloric requirement	gm/day/m ²	%	
5	14	3.23	+ 0.19	- 2.17	100 ± 0.46 ²	0.74 ± 0.01
30	10	3.33	- 0.27	- 2.64	98 ± 0.95	0.72 ± 0.03
60	10	3.44	- 0.01	- 2.39	98 ± 0.67	0.71 ± 0.05
85	10	3.38	- 0.29	- 2.69	97 ± 0.22	0.71 ± 0.03
			50% Caloric requirement			
5	12	2.58	- 1.53	- 3.31	99 ± 0.29	0.70 ± 0.05
85	6	2.51	- 0.79	- 2.51	97 ± 2.0	0.69 ± 0.02
			25% Caloric requirement			
5	6	2.90	- 1.39	- 3.38	99 ± 1.0	0.70 ± 0.06
50	8	2.67	- 0.70	- 2.50	100 ± 1.0	0.64 ± 0.01
85	6	3.01	- 2.08	- 3.31	98 ± 2.2	0.41 ± 0.02

¹ True digestibility corrected for endogenous fecal nitrogen as determined by feeding a protein-free diet.

² Standard error = $\sqrt{\frac{\sum d^2}{n(n-1)}}$. This equation refers to all such data presented in this paper.

NE₀ is the excretion of nitrogen on a protein-free diet. It is apparent that dogs fed restricted diets utilize nitrogen in a normal fashion when the protein content is relatively low. Additional dietary protein results in a small positive balance which cannot be increased further no matter how much protein is included in the diet. Thus, the reduction of the nitrogen balance index indicates the utilization of dietary protein to supply needed calories.

The data of table 1 demonstrate that the nitrogen balance index of casein is not altered from normal by varying the fat content of the diet from 5 to 85% of the total calories when the caloric intake is 100 or 50% of the calories required to maintain body weight. However, when the intake was reduced to 25%, an increase of dietary fat resulted in decreased nitrogen balance indexes below normal values.

The data of table 2 illustrate the effect of dietary fat on the nitrogen balance obtained in dogs fed large amounts of casein and restricted caloric intakes. In dogs fed 100% of the daily caloric requirement, nitrogen balance is not altered from normal by varying the fat content of the diet. At 25%, nitrogen balance is reduced slightly as is to be expected from the data shown in figure 1. The reduction of dietary calories results in a greater excretion of nitrogen in animals fed a protein-free diet, but the incorporation of large amounts of fat in the diet has no further effect. The nitrogen balance indexes were not calculated for the data obtained in dogs fed diets restricted in calories, due to the curvilinear nature of the nitrogen balance index equation. When sufficient calories are given, increasing the fat content of the diet does not alter the index.

The recent report of Swanson ('51) indicates that in rats previously depleted of protein, the removal of fat from restricted diets leads to an increased excretion of nitrogen. The protein sparing effect of fat may, however, vary with the physiological state of the animal. Protein depletion, for example, results in an imbalance of tissue proteins and enzyme systems which can alter the physiology of the animal (Alli-

TABLE 2
*The effect of dietary fat on the nitrogen balance index of casein obtained on dogs fed 0.30 gm casein
 nitrogen/day/kilogram body weight*

FAT CONTENT OF DIET	NO. OF DETER- MINATIONS	ABSORBED NITROGEN	NITROGEN BALANCE	PROTEIN- FREE NITROGEN BALANCE	DIGESTIBILITY ¹	NITROGEN BALANCE INDEX
% Cal.		gm/day/m ²	gm/day/m ² 100% Caloric requirement	gm/day/m ²	%	
5	8	5.16	+ 1.91	- 1.97	98 ± 1.1 ²	.75 ± .02 ²
63	7	4.95	+ 1.46	- 1.82	98 ± 0.91	.66 ± .03
85	7	5.11	+ 1.93	- 1.61	97 ± 0.90	.69 ± .02
			25% Caloric requirement			
5	6	6.05	+ 0.80	- 2.76	99 ± 0.61	...
63	6	6.02	+ 0.32	- 2.94	98 ± 0.57	...

¹ True digestibility corrected for endogenous fecal nitrogen as determined by feeding a protein-free diet.

² Standard error.

son, '51). The experiments reported here were performed with well nourished animals, and it is conceivable that different mechanisms may be operative in these animals as contrasted with those depleted of protein. These and other dietary factors which may affect the preservation of body tissues require further study.

Experiments by Munk and Rosenheim (Van Noorden, '07) demonstrated that the digestibility of fats was greatly reduced when dogs were fed low-protein diets for 6 to 8 weeks. Post mortem examinations revealed severe lesions of the gastrointestinal tract which might account for the loss of digestive powers. On the other hand, Coffey et al. ('40) found only slight variations in the digestibility of fats in dogs, and Barnes et al. ('44) found in rats that readily digestible foods such as lard are not affected in digestibility by the protein content of the diet. In the experiments reported here, the digestibility of protein is not altered from normal under any of the experimental conditions as shown in tables 1 and 2. The digestibility of fat, presented in table 3, is also normal in dogs with adequate protein stores. However, in dogs depleted of protein, fat digestibility is reduced from 98% to 88%. In no instance was any evidence of gastrointestinal disturbances observed with the high-fat diets.

Rosenthal and Allison ('51) have recently demonstrated the effects of caloric restriction on nitrogen retention. Two responses to a restricted caloric intake were recognized. The first response was an increase in the excretion of urinary nitrogen without any change in the nitrogen balance index of the dietary protein. The second response was a decrease in the nitrogen balance index reflecting a shift in the mechanism of nitrogen metabolism. They also indicated that resistance to caloric restriction was correlated, in part, with the magnitude of the protein stores of the body. As pointed out by Elman ('37), the body tissues may furnish a good deal of the daily caloric needs when the diet is restricted. Further studies to be described in this report indicate that

TABLE 3
Average digestibility of fat (lard) in normal and protein depleted dogs

NO. OF DETER- MINATIONS	ALBUMIN GLOBULIN	FAT CONTENT OF DIET	FATTY ACID INTAKE	FATTY ACID EXCRETED	DIGESTI- BILITY ¹
5	1.39 ± 0.11 ²	% Cal.	$gm/m^2/day$	$gm/m^2/day$	%
		0	0 \pm 0 ²	0.79 ± 0.02 ²	...
		30	39 ± 2	1.21 ± 0.26	99 ± 0.01
		60	79 ± 3	2.66 ± 0.15	98 ± 0.01
4	0.71 ± 0.03	85	115 ± 6	2.77 ± 0.39	98 ± 0.01
			Depleted		
		0	0 \pm 0	1.11 ± 0.6	...
		30	40 ± 3	8.12 ± 2.6	82 ± 2.9
		60	82 ± 5	11.2 ± 2.3	88 ± 2.1
		85	118 ± 5	14.7 ± 2.9	88 ± 1.5

¹ True digestibility corrected for endogenous fecal fat as determined by feeding a fat-free diet.

² Standard error.

the magnitude of the caloric reserves (body fat) also plays a role in the resistance of dogs to caloric restriction.

The data in figure 2 were obtained on two dogs fed a protein-free diet (white bars) alternating with protein feeding (slanted bars). The alternate feeding of protein-free and protein diets was done to make the calculation of nitrogen balance indexes possible, and to study, through protein-free

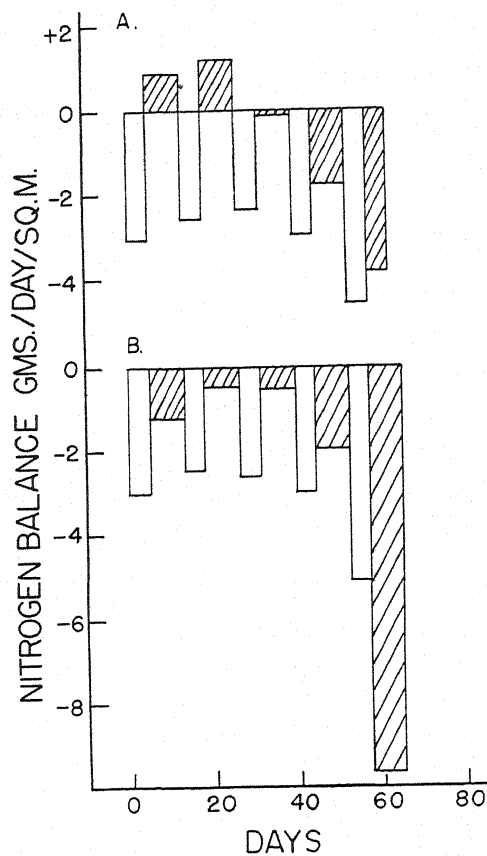


Fig. 2 Nitrogen balance versus days fed 25% of the caloric requirement. The white bars represent protein-free feeding periods and the slanted bars represent protein periods. Dog A was fed 0.30 gm casein nitrogen per day per kilogram of body weight and Dog B was fed 0.15 gm casein nitrogen per day per kilogram body weight.

feeding, the effects of caloric restriction on body nitrogen. The caloric intake was limited to 25% of the daily requirement and the fat content of the diet was maintained at 5% of the calories. Dog A was fed 0.30 gm casein nitrogen per day per kilogram of body weight, an amount sufficient to place the animal in positive balance. Dog B was fed 0.15 gm casein nitrogen per day per kilogram, which was not sufficient to maintain nitrogen equilibrium.

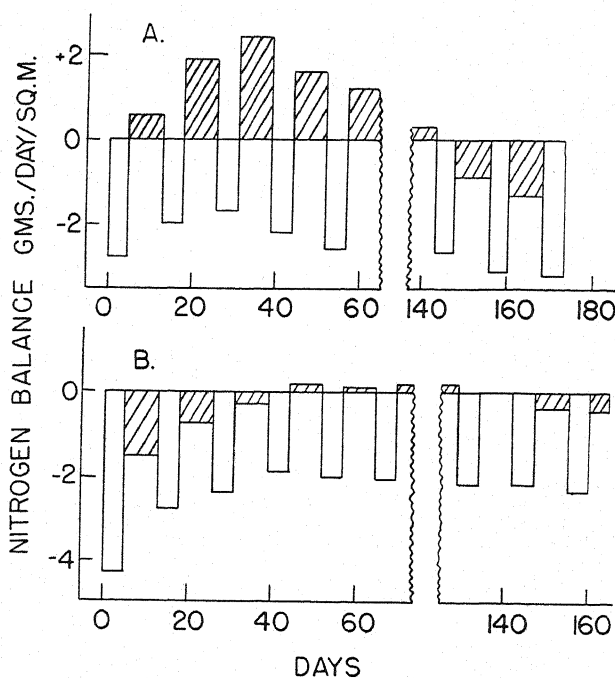


Fig. 3 Nitrogen balance versus days fed 25% of the caloric requirement for two dogs very resistant to the effects of caloric restriction. The data are expressed as in figure 2.

The reduction in excretion of nitrogen, shown by a gradual increase in positive nitrogen balance, could be expected as a result of the depleting effects of periods in negative nitrogen balance plus the effects of restricted caloric intake. The depletion phase was followed by an interval in which the excretion of nitrogen reached high values, and the animals

became quite ill. The data in figure 3 pertain to two dogs which were very resistant to the depleting effects of a caloric restriction, and therefore required many days to reach a state of negative nitrogen balance. These data have been selected to demonstrate the extreme responses of animals to partial starvation.

The effect of feeding restricted diets on the body weight of dogs is illustrated in figure 4. The ratio of the actual weight to the probable weight makes possible a common basis

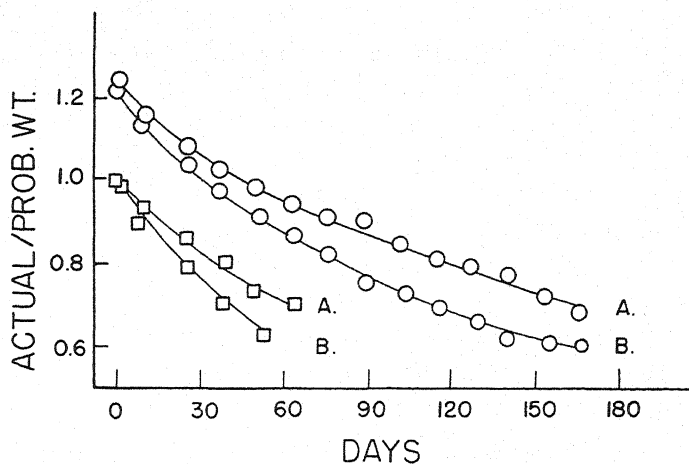


Fig. 4 The ratio of the actual weight to probable weight versus days fed 25% of the caloric requirement. The squares (□) represent the weight loss in the dogs shown in figure 2, and the circles (○) represent the dogs shown in figure 3.

of comparison between animals. Although the rate of loss in weight is similar in all animals, the heavier dogs have more reserves, and it requires a longer period of time to deplete them.

The nitrogen balance index is not altered while the tissue reserves are adequate but is reduced markedly during the shift toward negative nitrogen balance (Rosenthal and Allison, '51). The shift toward negative balance has been interpreted as one of utilization of both body and dietary nitrogen for energy purposes, leading to marked tissue breakdown.

SUMMARY

These experiments demonstrate that the incorporation of large amounts of fat in the diet does not alter the utilization of dietary protein when the caloric intake is at an optimal level. Nitrogen utilization may be reduced, however, when large amounts of fat are fed in conjunction with diets restricted in calories and protein. The addition of large amounts of protein to calorically deficient diets results in the use of some of the protein for energy purposes but prevents an increased excretion of nitrogen caused by high-fat diets. The average dog can digest large amounts of protein and fat, but digestibility of fat decreases with depletion of body protein. When dogs are subjected to caloric deprivation, they may be maintained in positive balance for variable lengths of time provided the quantity of dietary protein is sufficient and the caloric reserves of the body are adequate. Continued caloric restriction, however, eventually leads to marked loss of weight and an increase in the excretion of body and dietary nitrogen associated with severe tissue destruction. The role played by the caloric reserves of the body on the resistance of dogs to caloric restriction are discussed. The data also indicate the need for careful evaluation of nitrogen balance studies so that the variables of physiological state and composition of the diet are comparable.

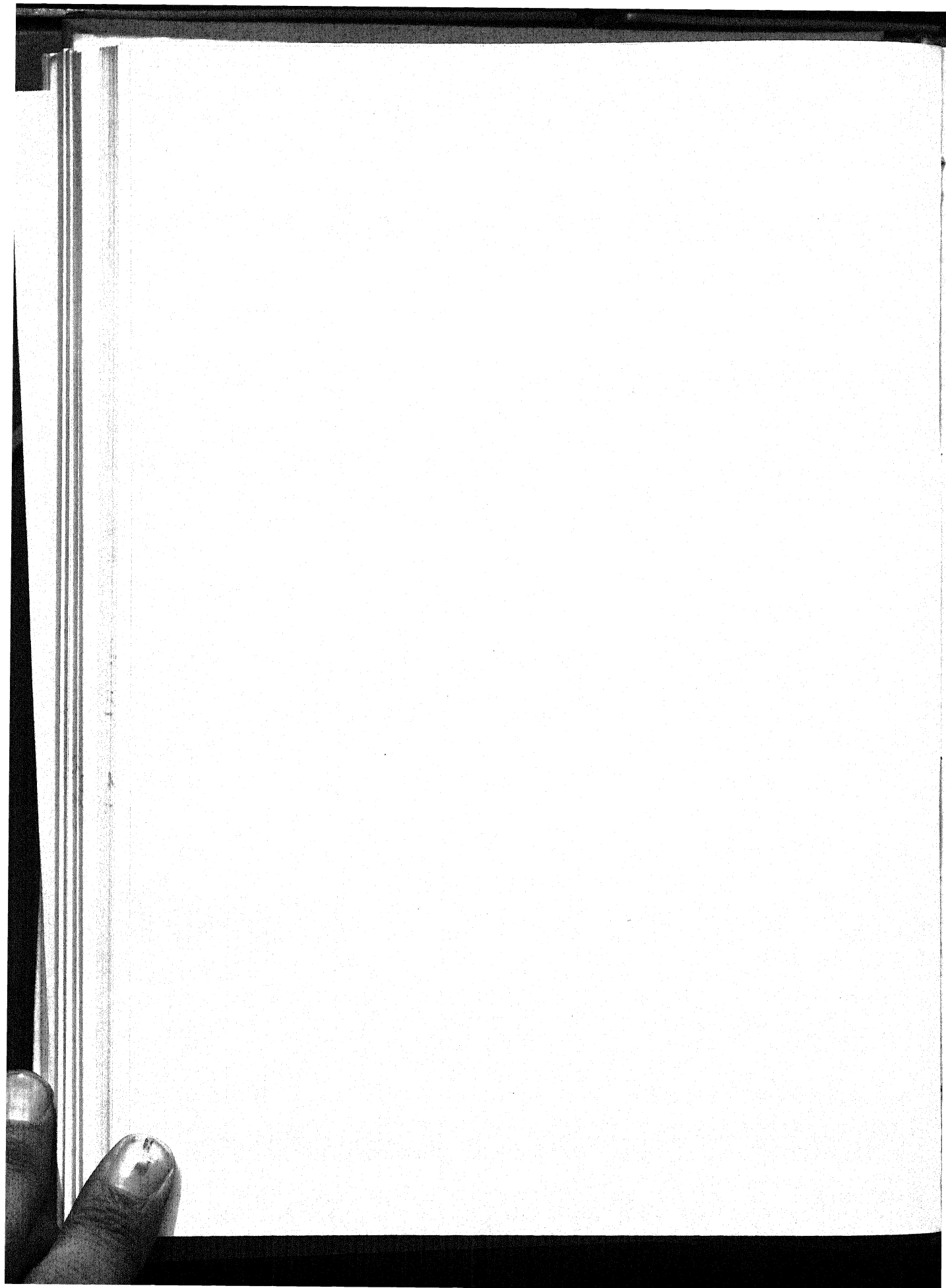
ACKNOWLEDGMENT

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BASAL HEAT PRODUCTION AND
ELIMINATION OF THIRTEEN NORMAL WOMEN
AT TEMPERATURES FROM
22°C. TO 35°C.¹

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SIXTEEN FIGURES

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It is well known that women have much more influence than men when it comes to adjusting room temperatures. That is why it seems strange that they have been neglected in studies of heat loss and the mechanism of temperature regulation. Many investigators have studied men, few have included women.

It is this neglect of the better half of the population that started our calorimeter studies of normal women in 1938, continued with war interruptions until 1948. Soon the reasons for the previous avoidance of women became apparent. It is difficult to find busy young women who can interrupt their work for a long series of tests extending over

¹ Clinical Calorimetry Paper, no. 55.

² G. F. Soderstrom died in October, 1948, at the age of 73. His services to calorimetry had been noteworthy. He built the Sage respiration calorimeter in 1912 and for 35 years operated the control table. He devised and constructed many new pieces of apparatus, these, like his techniques, always a little better than necessary. He took an active part in all experiments and he was the joint author of many papers.

a period of several months. Many young women, otherwise perfectly normal, have irregularities in the time of menstruation and occasional anovulatory cycles. Also, women show rather large individual differences, and it happened that the group of 6 normal women studied before the war had, on the average, a greater response to cold than the 7 women studied in the second series after the war. Since a much better picture is given by a group of 13 than by 6 or 7, the figures for all 13 women are assembled in this paper, even though some of the data have been published in previous reports (Hardy and Milhorat, '39; Hardy and DuBois, '40; Hardy, Milhorat and DuBois, '41; Hardy, Shorr and DuBois, '47; Hardy, Ebaugh, Stevens and DuBois, '50). The technique in the two series was identical, and it has been described in papers 49 to 53 of this series (Clinical Calorimetry, '38). The women came into the laboratory at about 8:30 A.M. A nurse helped them undress at about 9, weighed them and then put them in the darkened Sage Calorimeter. The box was sealed and after a preliminary period of about three-fourths of an hour the first experimental period ("First Hour") was started. Most of the experimental periods began between 10:15 and 11 A.M. During the preliminary period the women measured the skin temperatures in 20 places using the Hardy radiometer. This only required two or three minutes and involved the expenditure of one or two cal. Immediately after the end of the experiment they measured the surface temperature again. In many experiments, as is shown in the figures, skin temperatures were measured at the beginning of the second and subsequent periods. For this reason the estimates of the basal rates in table 1 are based on the data for the first hour only.

The observations at different calorimeter temperatures were run in random order, but as a rule the first was in or near the comfort zone. Since the tests had to be planned a week or two in advance it was impractical to limit them to any one phase of the menstrual cycle.

The women were instructed to observe and report any discomfort, sensations of cold or warmth, tensing of the muscles and the exact time of onset of shivering. At the end of the experiment they recorded their sensations in detail. All were cooperative but a few could not complete the series in the cold on account of changes in work schedules. In most experiments there were two periods of one hour each. Sometimes periods longer or shorter than an hour were used on account of technical difficulties or approaching chills. In some cases there was only one basal hour followed by factors such as alcohol or cold water, the effects of which will be reported in subsequent publications. Some of the best subjects could stand experiments lasting from three to 5 hours.

With well-trained subjects the basal figures obtained in the Sage respiration calorimeter are practically identical with those found using the Benedict type of closed circuit apparatus in 6-minute periods. Subjects not so well-trained find it difficult to remain motionless two or three hours and the metabolism tends to rise, perhaps on account of fatigue or slight restlessness. Several experiments have been excluded from this series because it was noted at the time of the experiment that the conditions were not "standard" or basal. The women in the calorimeter could be watched; movements were detected promptly by a slight rise in air temperature and sudden elevation of the small spirometer on the top of the calorimeter. A work adder was attached to this spirometer giving a record of all movements during each hour. In previous years it had been shown that this was delicate enough to record the movement of a man when he twisted his mustache.

EXPERIMENTAL

Subjects

Table 1 shows the data for the subjects. All were given careful physical examinations and every individual was found to be in good health. The records of the women provide more details. The experiments are listed with their dates in the order of rising temperatures to make easier comparison with the graphs.

Subjects 1, 2, 4a, 5, 6 and 7 have the same numbers as in our previous reports. Graphs of subjects 2 and 4a (Hardy and DuBois, '40) and data of subjects 1 and 2 with averages of subjects 1 to 7 (Hardy, Milhorat and DuBois, '41) have been published. These data are now recalculated in slightly altered form with more emphasis on the differences between the first and second experimental hours.

Number 3, J. W., of the first series has been omitted from this report on account of her amenorrhea. The 1940 series of A. S. (4b) is published for the first time.

Number 1, H. G., is a research associate who has been the subject of many kinds of experiments. Calorimeter temperature was 23.1 on February 2, 1938, 15 days after menses. She had taken a small early breakfast of the type that causes little or no increase of metabolism. First hour cold and tense, second

TABLE 1
Normal women subjects

NO.	INITIALS	AGE	AV. WT.	HEIGHT	SURFACE AREA	CAL. SQ. M/HOUR CONF. ZONE FIRST HR.	NO. OF EXP.	
							Comfort zone	Total
			<i>kg</i>	<i>cm</i>	<i>sq. m</i>			
1	H.G.	35	64	175	1.77	32.4	2	6
2	P.N.	25	60	162	1.62	30.7	5	10
4a	A.S. 1939	21	76	162	1.82	28.8	5	13
4b	A.S. 1940	22	64	162	1.69	28.1	2	8
5	H.E.	23	56	168	1.63	32.3	2	7
6	E.M.	42	53	169	1.61	33.1	1	3
7	A.B.	26	54	165	1.59	30.2	3	3
8	M.S.	24	61	175	1.75	34.1	3	3
9	B.R.	21	62	161	1.65	30.6	3	6
10	G.C.	28	78	176	1.93	27.6	2	4
11	P.H.	22	55	163	1.58	32.1	2	4
12	H.H.	24	40	154	1.34	34.5	1	1
13	L.T.	23	51	163	1.53	33.5	3	4
14	R.C.	23	76	172	1.88	29.3	3	4

period slight shivering. Temperature was 23.3 on February 9, 1938, 22 days after menses. Temperature was 25.2 on January 19, 1938, one day after menses. In the second and third hours she was cold and had a desire to urinate. In the third period there was slight shivering. Temperature was 26.7 on January 12, three days before start of menses. Temperature was 30.3 on March 11, 1938; second day of menses, no discomfort. Temperature was 33.7 on March 2, 1938, 8 days before menses, had a small early breakfast. First hour warm and drowsy; second hour, a slight headache.

Number 2, P. N., is an artist's model, and is married. Menstrual data not available. Calorimeter temperature was 24.0 on March 25, 1938. Period terminated 20 minutes after start on account of an approaching chill. Tempera-

ture was 24.9 on January 21, 1938. In the third period there was a mild chill. Temperature was 26.8 on January 14, 1938. Temperature was 28.3 on February 16, 1938. Temperature was 29.8 on February 18, 1938. Temperature was 31.2 on March 18, 1938. Temperature was 32.0 on February 25, 1938. Temperature was 35.5 on March 4, 1938. By 11:30 she was sweating all over the body. Temperature was 35.8 on March 8, 1938. Slept for a few minutes in first period. Sweating profusely and slightly restless. Felt cooler towards end of the experiment.

Number 4a, A. S., a research associate who has served as the subject of many experiments. She could remain quiet in the calorimeter for long periods. During the first year of experiments in 1939, her weight varied between 75.2 and 76.5 kg. During the summer she decided that she was too heavy and reduced her diet until she had lost about 12 kg. On December 7, 1939, when the second series (unpublished in our first reports) started, she weighed 63.8 kg. She maintained an uncomfortably low diet and when the experiments ended in May, 1940 she weighed 64.5 kg. Her menstrual intervals were about 28 days. Her data for 1939 and 1940 are given below.

Nineteen thirty-nine series. Calorimeter temperature was 22.4 on May 16, 4 days after menses; she was cold and on the verge of shivering during the last few minutes. Had a chill on coming out of the calorimeter. Temperature was 22.6 on April 6, 18 days after menses. Temperature was 23.5 on May 23, 11 days after menses. Temperature was 24.2 on March 9, 18 days after menses. Temperature was 24.9 on May 18, 6 days after experiment. Doubtful slight shivering in second hour. Temperature was 27.9 on March 10, 19 days after menses. Temperature was 30.0 on May 9, two days before menses. Temperature was 31.0 on May 24, 12 days after menses. Sweating in axilla and palms. Temperature was 31.7 on March 23, 5 days after menses. Temperature was 33.1 on March 30, 12 days after menses. Temperature was 34.9 on April 20, 5 days after menses.

Nineteen forty series. Number 4b, A. S. Data on menstrual periods lacking. Calorimeter temperature was 22.1 on February 8. Tense during last half hour. Chilled slightly on coming out of calorimeter. Temperature was 22.2 on February 14; humidity 70%. This experiment and those at 22.3, on February 6, and 22.4 on January 23, were not used in averaging percentage losses by radiation convection and vaporization because the humidity was high in all three experiments. In the first two, the calorimeter felt stuffy. At 22.4, with the humidity 55% to 59%, she did not complain of stuffiness. Temperature was 23.9 on December 12, 1939. Temperature was 27.8 on January 31, 1940; humidity 50% to 57%; comfortable. Temperature was 27.9 on March 10. Temperature was 33.3 on May 22.

Number 5, H. E., is a housewife. This young woman had been examined the previous year in the dispensary on account of loss of weight and anemia. Her thyroid was slightly enlarged. In 1939 she was in good health. There was no evidence of constitutional disease and her weight loss was ascribed to frequent colds and long hours of work. The menstrual intervals were 29 to 30 days. Calorimeter temperature was 22.6 on March 16. Chilly, relaxed, and no shivering. Menses started April 9. Temperature was 24.3 on March 21. Temperature was

26.0 on April 18. Temperature was 27.5 on March 28. Temperature was 29.9 on March 17. Temperature was 32.0 on February 17. She was "hot but not uncomfortable," and perspired a little. Temperature was 33.9 on April 4. First hour warm, but not uncomfortable. Second hour, hot, uncomfortable, and sweating profusely.

Number 6, E. M., is a professor of physiology, who has lived most of her adult life in India, and has been the subject of many experiments. Her basal metabolic rate in Madras averaged 28.5 cal. per square meter per hour. Recently in New York it was reported as 32.2. Her hemoglobin was 65%. She has always been susceptible to cold. Calorimeter temperature was 24.7 on February 20, 1940. Feet cold, and slight twitching of muscles. She had mild shivering after she came out of the calorimeter. Temperature was 26.4 on February 21. Chilly, and slight twitches of muscles; goose flesh. She shivered when she came out of calorimeter. Temperature was 32.4 on February 16. Comfortable.

Number 7, A. B., is a housewife. Calorimeter temperature was 28.3 on April 12, 1938, one day before menses. Temperature was 30.0 on April 19, 4 days after menses. In these two experiments the skin temperature measurements were unreliable so that it was impossible to measure radiation and convection. Temperature was 31.9 on April 21. Temperature was 32.0 on May 10, second day of menses. In all of these experiments she was comfortable and quiet.

Number 8, M. S., is a technician. Her general health was excellent, but she was losing weight. At the end of the experiments a careful study in the clinic was negative. Her menses were regular with little discomfort. Calorimeter temperature was 28.1 on January 19, 1939, 8 days before menses. First hour warm and comfortable, second hour slightly cold; quiet. Temperature was 30.1 on January 26, day before menses. Temperature was 30.2, last day of menses. No discomfort.

Number 9, B. R., is a student nurse. She was in good health but her weight was fluctuating as a result of dieting. Her menstrual interval was 21-28 days. Vaginal smears in June, 1948, showed an anovulatory cycle with poor follicular activity. Maximal cornification was 1%. Most smears showed predominantly large nucleated superficial cells and cytolysis. Calorimeter temperature was 22.2 on May 15, 1947, 61.7 kg, 7 days after menses. During the last 10 minutes of the experiment she was tense. Five minutes after the end of the experiment she shivered all over her body. Temperature was 24.0 on June 19, 60.0 kg, last day of menses. Temperature was 26.1 on May 8, 61.4 kg, third day of menses. Temperature was 30.0 on May 13, 61.2 kg, 5 days after menses; a little too warm, and slight sweating. Temperature was 31.7 on May 22, 62.5 kg; 14 days after menses; a little too warm and slight sweating.

Number 10, G. C., is a dietitian who has lived in the South until she came to New York 5 months ago. She "feels the cold" more than her companions. Up to 9 months previous to the experiments she had been given thyroid intermittently on account of overweight and one low BMR. At the time of the experiments there was no evidence of hypothyroidism except for a low basal. Her menses which are normal began on March 6 and on April 4, 1948. Vaginal smears showed ovulation between April 16 and 18. Calorimeter temperature

was 24.4 on March 31, 1948. Cold and tense, but not shivering. Temperature was 26.8 on March 24; felt slightly cool the first hour. Temperature was 30.0 on March 23; slightly cool. Temperature was 30.7, and she felt very comfortable.

Number 11, P. H., is a nurse. Weight stable. Menstrual periods began March 29 and April 25, 1948. The vaginal smears during the experimental period showed an anovulatory cycle with good follicular activity reaching maximal cornification at 60% on April 16. Calorimeter temperature was 24.9 on April 15, 1948. Cold but relaxed. Temperature was 30.1 on April 13. Felt comfortable. Temperature was 31.3 on April 7. Uncomfortably warm, but relaxed with slight sweating in axillae.

Number 12, H. H., is a nurse of Polish descent, petite, weight stable. In 1947 she had dysmenorrhea for three months, and she was studied in the Endocrine Clinic through two ovulatory cycles. The vaginal smears showed maximal cornification of 40% and 25%. The luteal phase was normal. In 1948, at the time of the experiments, the menses were regular with a 32-day cycle. A menstrual period began March 26. Temperature was 25.9 in April, 1948; felt cool. The experiments were terminated by the news of the death of her brother.

Number 13, L. T., is a nurse. Normal menstruation history, menses began on April 22 and on May 24, 1948. The May 25-June 25 cycle was followed by vaginal smears and morning basal temperature. Ovulation probably occurred June 12-13 with maximal cornification on June 12. The temperature curve began to rise June 12. Calorimeter temperature was 24.0 on May 18, 1948; cool, no inclination to shiver. Temperature was 26.2 on May 13. Hands and feet cold, otherwise comfortable. Temperature was 28.2 on May 11; comfortable. Temperature was 31 on May 20.

Number 14, R. C., is a nurse. She was somewhat overweight and her weight remained constant during the last year. Four years ago she had received thyroid therapy but there is no evidence of hypothyroidism. Menses started on May 13 and on June 6, 1948. Vaginal smears showed ovulation between June 22 and 24 and there was a distinct rise in temperature at this time. Smears showed maximal cornification 60%. The luteal phase was poor with scant secretion and little mucus. Calorimeter temperature was 24.7 on June 3, 1948. Cold, had waves of chilliness and goose flesh but did not shiver. Temperature was 26.5 on May 27. Feet cold. Temperature was 28.0 on May 25. Temperature was 30.9 on June 1. Quiet, axillae and forehead moist.

Figures 1 and 2 give a general picture of the changes in metabolism in calorimeter temperatures from 22°C. to 35°C. Inasmuch as the calorimeter walls are in thermal equilibrium with the air, these are equivalent to the "operative" temperatures of Winslow, Herrington and Gagge ('37). The points on the curves give the average of the first and second hours except in those cases where only the first hour was

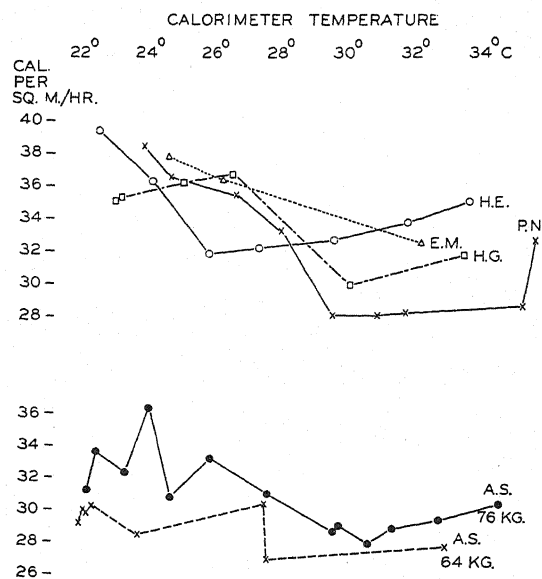


Figure 1

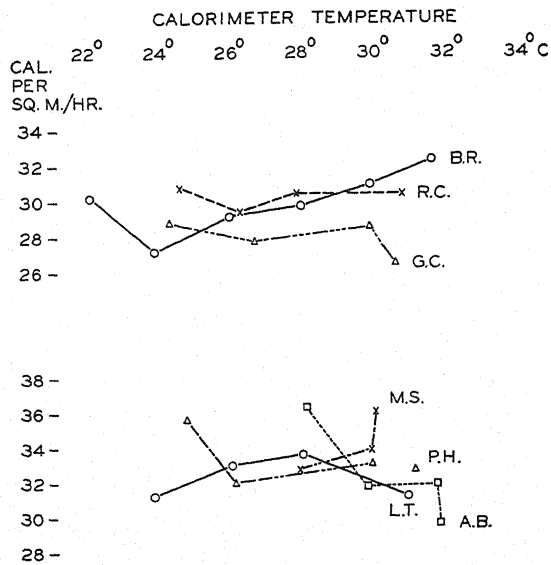


Figure 2

Figs. 1 and 2 Basal metabolism at calorimeter temperatures from 22-35°C. averages of first and second hours except in a few instances when second hours were not available.

basal. Individual differences are brought out clearly. The first graph shows 4 women whose metabolism was high in the cold zone, the second shows the metabolism for A. S. in 1939 at 76 kg, and the lower and flatter curve in 1940, when she was on a reducing diet.

Figures 3-8 give the details of the 5 women who were studied in 6 or more experiments. Figure 9 charts the respiratory quotients in all of the experimental hours. All are in the range to be expected from the previous diet and length of fasting.

Figure 10 is a smoothed curve giving the heat production calculated from oxygen consumptions in the first and second hours. Seventy-six experiments on 13 women were available. The need for smoothing is seen from the irregular distribution of measurements over the temperature range. For example, there were only two experiments between 25.0 and 25.9, but 11 at 24.0 and 24.9, and 10 at 26.0 and 26.9. The average level for 25° was therefore determined by adding the totals for 24°, 25°, and 26° and dividing by 23. The whole curve was smoothed by using three degrees except at the extremities where only two degrees could be employed. Trial curves were made using the averages of all experiments but it became evident that this was unfair. For example, the 6 experiments on A. S. far outweighed the one for H. E. plus one for B. R. The curves in figure 10 were obtained by using the averages of the individuals, e.g., three at the 22° level.

*Heat production of women at different
environmental temperatures*

From an inspection of figure 10 it is apparent that the metabolism is distinctly higher in the second hour than in the first hour. There is also evidence that in the first, or basal, hour the metabolism is lowest in the region of 30-32 with a tendency to be higher in the warm zone and distinctly higher in the cold zone. The two extremities of the curves do not show these trends. Only three individuals, H. E., A.

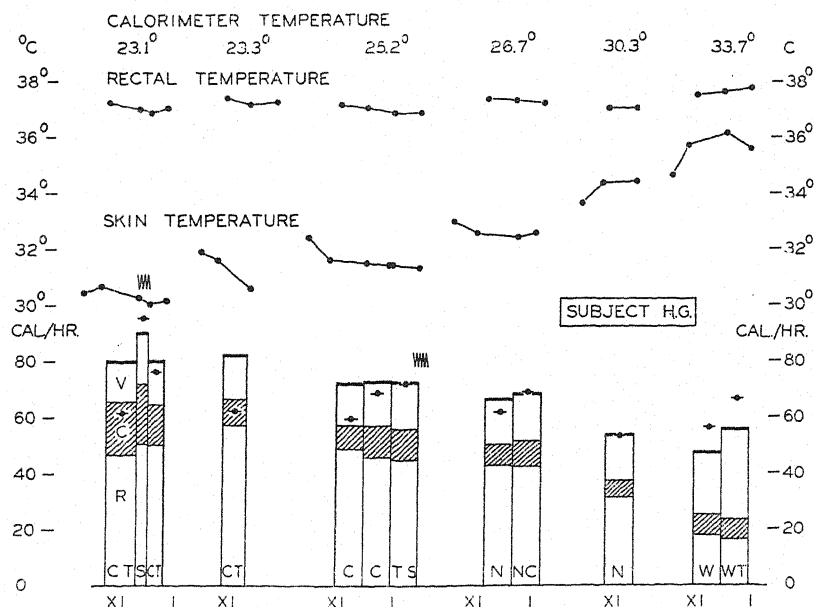


Figure 3

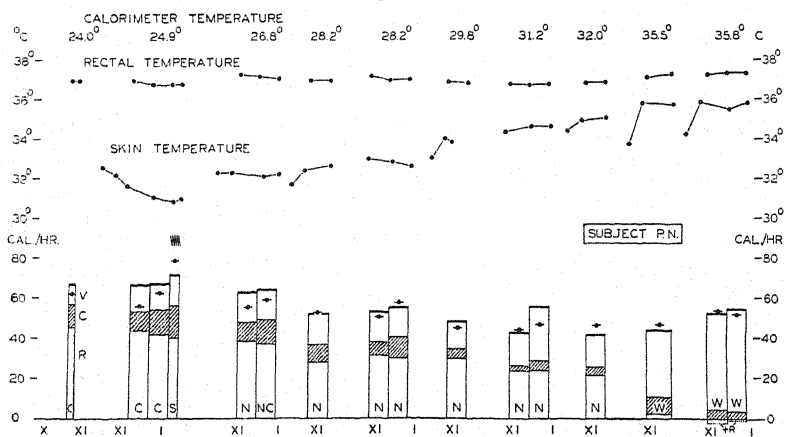


Figure 4

Figs. 3-8 The top line gives the average temperature of the calorimeter at the time of the experiment. The skin temperatures are the average of readings of each part of the body weighted according to the effective radiating area of the part. The black dots with short horizontal bars represent the heat production as determined by the method of indirect calorimetry. The columns show the total heat loss as measured by direct calorimetry. The top division of each column gives the loss by vaporization (V); the hatched portion by convection (C); and the lowest division, radiation (R). At the base of each column letters indicate the sensations of the subjects: S, shivering (also marked by zigzag lines above the column); C, cold; CT, cold with tension in a few muscles; N, neutral, or comfortable; W, warm. The Roman numerals at the base show the time of day.

S. and B. R., were studied between 22 and 23.9. Their basals in the comfort zone averaged 2% below the graph as a whole. In the range from 34 to 35.9 there were only two, A. S. and P. N., with an average basal 4% below the group. The preponderance of women with low metabolism at the two extremities accounts for part, if not all, of the changes in the direction of the curve.

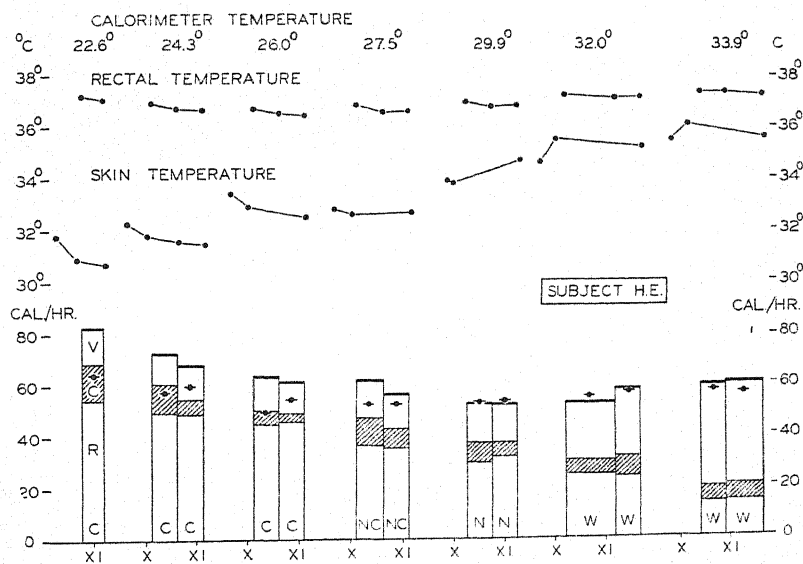


Figure 5

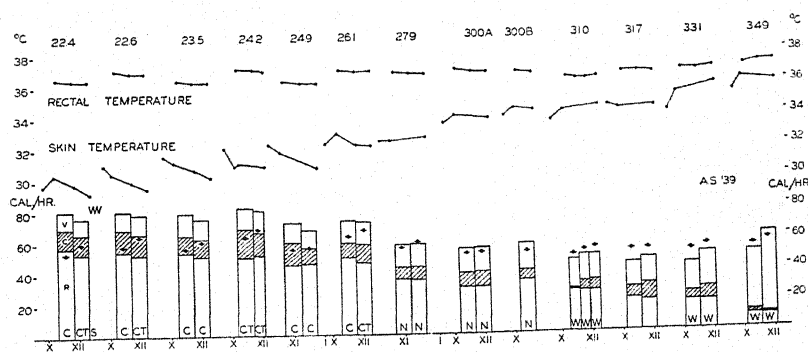
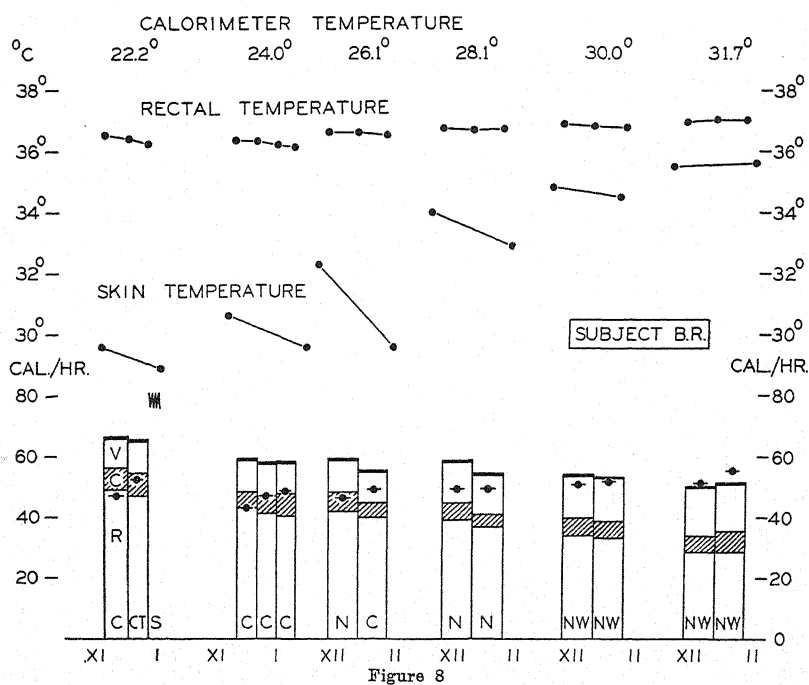
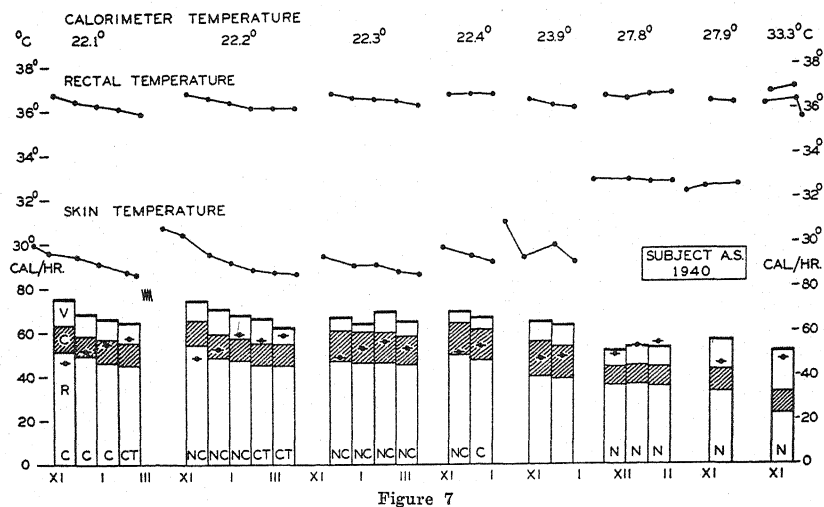


Figure 6



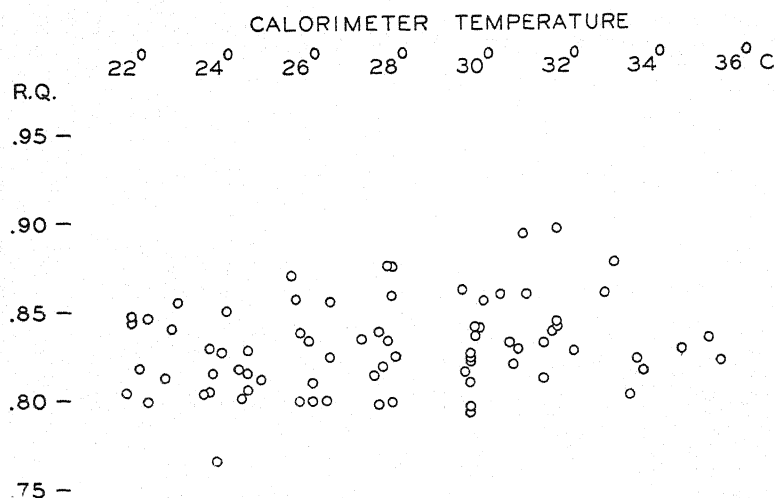


Fig. 9 The average respiratory quotients, in most cases the average of two experimental hours. Three experiments have been omitted on account of technical difficulty in measuring oxygen. One was omitted because the woman had eaten coffee cake at 9 the previous evening. The average quotient indicates that the calories derived from carbohydrate and fat were about equal. The protein calories were low.

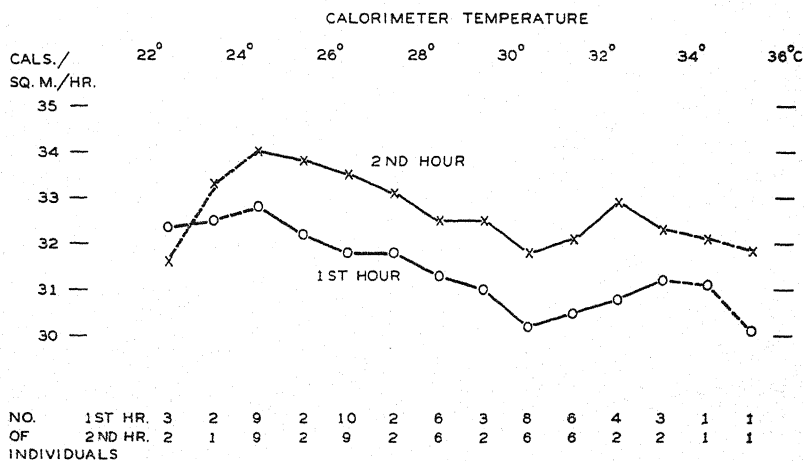


Fig. 10 Smoothed curve giving the average heat production in terms of calories per square meter per hour. The circles represent the first hours only, the crosses the second hours. The average metabolism of each individual is averaged with those of other individuals at the same and at two adjacent temperatures.

Rise of heat production in the second hour

The causes for the rise of metabolism in the second hour are not entirely clear. In some, but not in all, of the second periods, there was the factor of measuring the surface. There is also the factor of increased length of exposure to an uncomfortable environment with boredom and slight, undetected, restlessness. In the cold and warm zones there is the increased effect of adjustment to the environment, the factor we are trying to measure.

In the series of 13 women studied in 1938 and 1948 the rise of metabolism in the second hour averaged 5.9% in the cold zone, 3.4% in the comfort zone, and 3.2% in the warm zone. In the range from 22-35 there were 43 experiments with second hours, and in 33 of these pairs, there was a rise in the second hour. Only 10 showed a fall. Statistically the chances of this occurring by accident are about one in 1,000.

This tendency towards a rise in metabolism in the second hour is an old phenomenon in the Sage respiration calorimeter. It is shown in the records of experiments made between 1913 and 1932 before the introduction of surface temperature measurements. The subjects, lightly dressed, were very comfortable in environments of 23 to 24. It was possible to find 31 satisfactory experiments on 19 normal men and two normal women. In 19 of these basal experiments the heat production rose in the second hour, in 12 it fell. Averaging all of these with regard to sign the figures indicate an average rise of 1.4%. In the case of the normal control, E. F. D. B., who was studied most often, the average rise before 1932 was only 0.6%. After 1936 when he was exposed naked to various temperatures, measuring the surface temperatures in some but not all of the experiments, the rise in the second hour averaged 3.0% in the cold zone, 3.1% in the comfort zone, and 4.3% in the warm zone.

Many others have noted a changing metabolism during the experimental periods. Berkson and Boothby ('38) have made

a careful analysis of their records with short periods using the Tissot technique. "For the first two or three observations (which will mean on the average until about 9:30 A.M.) there is a decrease in metabolism amounting on the average to about 0.4 cal. per square meter. Then there is a rapid and continuous rise to above the initial value so that by the 7th observation (about 12 noon) the mean increment of metabolism is positive and about 0.4 cal. per square meter. We may speculate that the initial lowering is correlated with an increasing adjustment to basal conditions and that as time passes, restlessness begins to be effective in raising the metabolism." They found that for the 8th and 9th observations the rise was about 1.0 to 1.4 cal. per square meter.

The differences of about 3% are not great and most investigators feel happy when two consecutive determinations of the basal or "standard" metabolism come within 5%. It is only the strong preponderance of increases in the second hour that inclines us to take the first calorimeter hour as the truer index of the basal. The average 5.9% rise of the women in the cold zone as opposed to a 3 or 4% rise in the other zones indicates, but does not prove, that the prolonging of the exposure to cold is a factor which increases metabolism. A study of the continuing adjustment of loss by radiation supports this view.

Body temperatures

Figure 11 gives the curves for average rectal and surface temperatures smoothed by averaging the results at each degree with those of the adjacent degrees above and below. The line for the average rectal temperature is surprisingly level at 36.8° to 36.9°C. from environmental temperature of 25 to 32. It is slightly lower below 25°C., and higher above 32°C. A study of the individual graphs shows a tendency for falling rectal temperatures during any experiment in the cold zone, a level or slightly falling in the comfort zone, and a rising or level temperature in the warm zone. The level or falling rectal temperature in the comfort zone con-

firms the statement of DuBois ('41) that the usual morning rise of body temperature is absent under strict basal conditions.

The rectal temperature measurements obtained by the Sage calorimeter technique are probably more uniform than those of any other series in the literature for several reasons. The

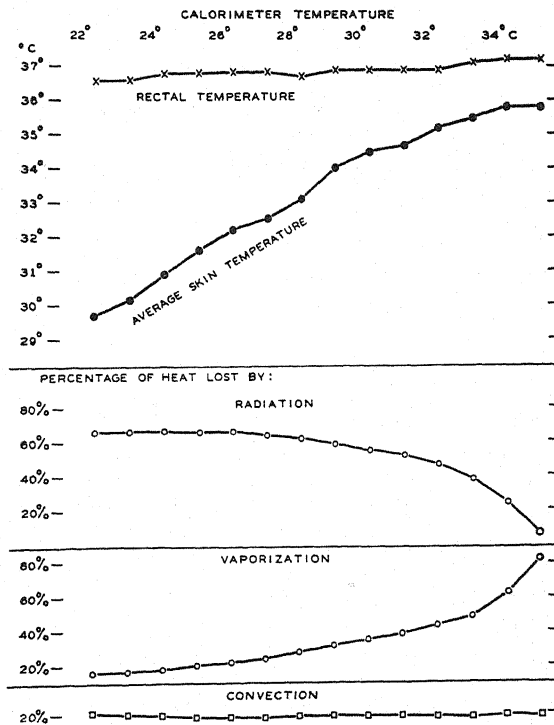


Fig 11 Smoothed curves giving averages in all experiments during the first experimental hour.

thermal element of 100 ohms resistance is encased in Woods metal in a silver tube 6 cm long and 8 mm in diameter. This is attached to a moderately flexible rubber tube and is inserted so that the tip is in the sigmoid flexure 12 cm from the anus. This is at the depth which, according to Benedict and Slack ('11), and Mead and Bonmarito ('49), gives the maximal readings. X-rays of the Sage thermometer taken with two

normal men have shown that it lies almost exactly in the center of the lower pelvis where it would be least influenced by blood flowing from the legs. There must be some influence from the cooled blood returning from the legs especially in the cold zone; it is impossible to measure exactly how much without using thermometers in the abdomen and thorax.

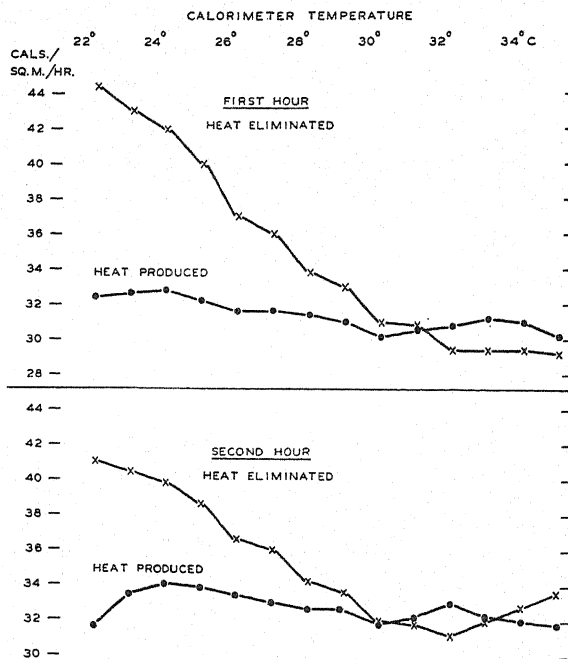


Fig. 12 Heat production contrasted with heat elimination in the first and second experimental hours.

Surface temperatures. Figure 11 shows the smoothed curves of average surface temperature with its steady rise in environments from 22°C. to 29°C., then a slower rise with a leveling at 34°C.-35°C. The graphs of individual experiments show falling skin temperatures in the cold zone, rising in the warm zone, with variable changes in the comfort zone.

These surface temperatures were measured by the Hardy radiometer in 20 parts of the body and were then weighted for the effective radiating areas of each region. A recent

careful comparison of surface thermometers (Stoll and Hardy, '49, '50) has shown that the radiometer is the most reliable instrument. There is of course a wide variation in the different parts of the body, as was demonstrated in the experiments on men (Hardy and DuBois, '38). The estimations of conductance through the peripheral tissues and the average body temperature have been discussed in the earlier reports on women (Hardy and DuBois, '40; Hardy, Milhorat and DuBois, '41).

Heat elimination

Figures 11 and 12 give the relationship of heat production, heat loss and the percentage of calories lost in radiation, convection and vaporization. Here again smoothed curves are used for both the first and second hours. Naturally in the cold zone a great deal of heat is being lost from the periphery of the body. In the first hour the heat elimination at 22°C. is 37% greater than the heat production, and at 26°C. it is 17% greater. It is only between 30 and 32.9 that they are approximately the same in the first hour. Above 32 the elimination lags behind the production and does not catch up until the second hour. It must be remembered that the first calorimeter hour starts one or two hours after the women have undressed and commenced their exposure to the particular environment. The loss of heat in the cold and the adjustments in the cold and warm zones change as the hours progress. This is shown by the fact that in the cold zone the heat eliminated in the second calorimeter hour is distinctly less than in the first hour in spite of an increased heat production. In the warm zone the heat elimination increased during the second hour, showing a tendency to balance the accumulation of heat in the first hour.

A study of the details of heat loss explains the mechanism of these changes in the second hour. At temperatures of 23 and above, the percentage lost by radiation decreases slightly with falling skin temperatures and the percentage lost by convection increases slightly. In the warm zone the percentage lost by vaporization increases during the second hour.

The smoothed curves in figure 11 demonstrate a surprising uniformity of the average loss by convection although the differences in individual hours are relatively large. The fact that the average rise of convection in the second hour is small indicates that the subjects had little increase in their movements during the second hour. A paper by Hardy, Milhorat and DuBois ('38) demonstrated the marked increase in convection with moderate exercise or chills.

When the environmental temperature is nearly the same as the skin temperature there can be little or no loss by radiation and convection. Vaporization takes care of all of the loss and in doing so keeps the skin temperature far enough below the core temperature to provide a gradient between the core and the periphery.

DISCUSSION OF RESULTS

Basal metabolism

The average basal metabolic rate of the 13 women with an average age of 25.6 years was 31.2 cal. per square meter per hour in the comfort zone, and it so happened that it was the same for the first and second series. The different individuals showed considerable variations in their averages. The lowest was G. C. at 27.6, the highest 34.5 in the first and only observation on H. H. The estimate of the average BMR for women of this age made by Aub and DuBois in 1917 was 37.0. In 1936 Boothby, Berkson and Dunn gave as standards 36.18 for the ages 20-24 and 35.70 for the ages 25-44. Since that time there have been many reports on normal women with figures that are much lower. The excellent new standards of Robertson and Reid ('52) give an average of 33.9. All of the women of this series may be considered to be within the normal range.

The effect of environmental temperature on basal metabolism

There are available in this series 76 experiments on 13 women and it is possible to make a more accurate analysis

of the effect of the environment on metabolism. The important points are whether or not an effect is demonstrable, and if so, the cause or causes of this effect.

The shapes of the average curves and the statistical analysis show a significant small rise of metabolism in the cold zone and a trend towards a rise in the warm zone. The causes are still uncertain.

The literature on the "chemical regulation" of Rubner and the effect of environment on men has been reviewed in our previous publications (Hardy and DuBois, '40; Hardy, Milhorat and DuBois, '41). The generally accepted view that there is no significant change in the basal metabolism of naked men in short exposures at temperatures from 22 to 35 is supported by much fragmentary evidence. It is, however, possible that if there were a study on men as comprehensive as this one on women, significant trends might be demonstrated. Unfortunately there are respiration calorimeter studies on only two normal men in this range of temperature (Hardy and DuBois, '38).

In addition to the publications quoted in our previous papers there are a few old studies and many recent ones that deserve attention. In general most writers support the view of little or no change in the metabolism of men in the range from 22 to 35. Almost everyone emphasizes the great variability in subjects. Some say that metabolism in the cold can rise without increase in muscle movements, others deny this. The recent German literature tends to include under Rubner's term "chemical regulation" factors such as chills and sweating whereas the Americans exclude these and limit the term to effects produced by hormones, emotions or effects similar to the specific dynamic action of foods.

Voit in 1878 was a pioneer in the study of cold. Loewy in 1890 published a fine study of men at different temperatures under basal conditions. There were 55 experiments. In 9 of these the oxygen consumption fell in the cold, in 20 it changed less than 5%. In 26 the oxygen rose in the cold. In half of these there was shivering or tension, in the other half no

shivering or tension was observable but Loewy believed that the rise in the cold should be ascribed to small twitches that cannot be detected by visible muscle contractions. Loewy recognized the importance of contraction of the peripheral blood vessels as a means of diminishing the loss of heat from the body. McConnell and Yagloglou in 1925 noted that the minimal metabolism of men was in the range between 75°F. and 83°F. "effective temperature." Horvath, Golden and Wagar ('46) placed a few men in air-conditioned rooms at different temperatures for two to 6 days. There was little evidence of any marked change in energy metabolism. Park and Palmes ('48) in a study of fever using a new and rapid type of calorimeter did not obtain data in favor of a "chemical regulation." All augmentations of heat production were caused by chills or were secondary to elevation of body temperature. In three out of their 4 graphs showing chills the metabolism remained level up to the onset of shivering. In the 4th case the body temperature and metabolism had risen slowly before the onset of chill.

In 1940 Dr. Eleanor Mason (E. M. of our series) studied in 21 women the effect of a change in residence from a temperate to a tropical climate. She found two types of women. Thirteen in type 1 showed a drop of 6 to 11% in the tropics. Eight in type 2 had little or no change. In 1944 Dr. Mason determined her own basal metabolism daily on a voyage from Honolulu to the tropics. Leaving Hawaii her BMR was 30.1-31.2 cal. per square meter per hour. Ten days later in the tropics it had fallen to 28.1. In 1938 Hick, Keeton and Glickman studied one man and 4 women at effective temperatures between 60°F. and 95°F. Judging from their graphs two of the women showed an increase in metabolism in the cold amounting to 6-10%, and all had an increase when very warm.

Important work on temperature regulation has appeared in the German literature. Thauer and Wezler ('43), using a climate chamber with rapid interferometer readings of oxygen and carbon dioxide, made 48 observations on three nor-

mal young men and one young woman for three-hour periods under basal conditions. The metabolism curve was level in their "comfort zone" 22°C.-38°C. Above 38, the heat production rose sharply. Below 22, the rise was variable depending on the amount of shivering. One man showed a curious dip of metabolism between 34°C. and 36°C. where the level was about 10% lower than at 28-30. Gollwitzer-Meyer ('37) had noted a similar drop in a warm bath that caused complete relaxation. The fall in metabolism in the one subject of Thauer and Wezler is about the same as the 10% drop during sleep noted by Mason and Benedict ('34). The woman subject in Thauer's experiments for the first hour at 15°C. had a distinct rise in metabolism without trace of shivering or chills. Wezler and Thauer ('43) compared the vaporization loss of one man and one woman at 50°C. and 50% humidity. The woman lost only 1.01 kg, the man 1.61 kg, and his curve of water loss started to rise sooner and was much steeper. König ('44), using the same apparatus, studied 8 more men naked or in swimming trunks. He found great variations. One of the men (Büttner) had no rise in metabolism in the room at 13°C. König believes that without muscle contraction there is no rise in metabolism. Wezler and Neuroth ('49) again using the same apparatus studied 4 men and two women but it is hard to interpret their results as the time of chills is not indicated. They tried to calculate the fall in metabolism that one would expect in the peripheral tissues as the peripheral temperature fell (Van't Hoff effect). They call attention to the fact that if there is a reduction in the peripheral zone there must be a chemical regulation increase in the core of the body.

Tensing and the approach to chills

Figure 13 reproduces three chill experiments on men from the report of Hardy, Milhorat and DuBois ('38). In all three experiments the metabolism remained at or near the man's average basal level up to the onset of the chill. Figures 3

to 8 show that the women had a rising metabolism in the hours preceding the chills.

When the body cools to a certain point there is an involuntary tensing of the muscles in the trunk or in one extremity after another. Often this ceases in a few minutes but if the body is cool enough and if the man cannot control him-

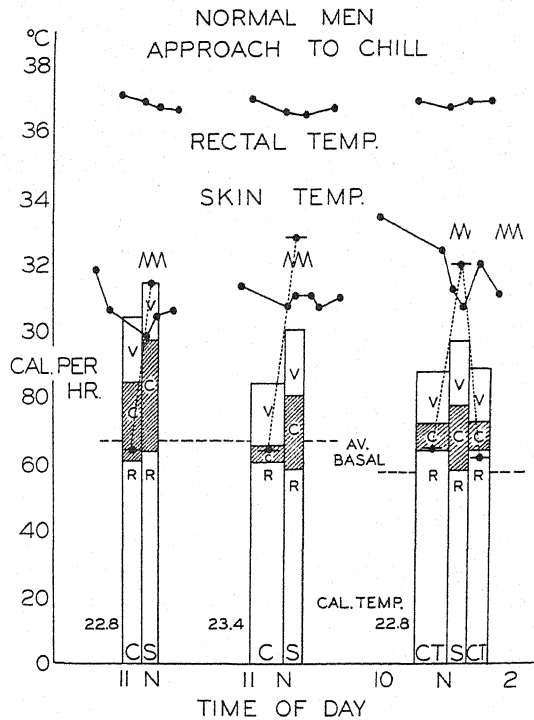


Fig. 13 Three calorimeter experiments on two normal men showing that the metabolism was close to the man's average basal in the period just before a chill.

self the mild tensing changes abruptly into frank shivering. The important points at question are: (1) does the subject always appreciate the onset of tensing; (2) can it always be detected by a careful observer; (3) can it be detected by instruments; and (4), and the most important, does it cause a significant rise in metabolism? Opinions are divided on all of these points.

We believe that our subjects, who were all intelligent and carefully trained, gave us accurate reports on the presence or absence of tensing. Almost everyone is agreed that it is often impossible for an observer to detect tensing since it may occur without visible movement of the extremity. The

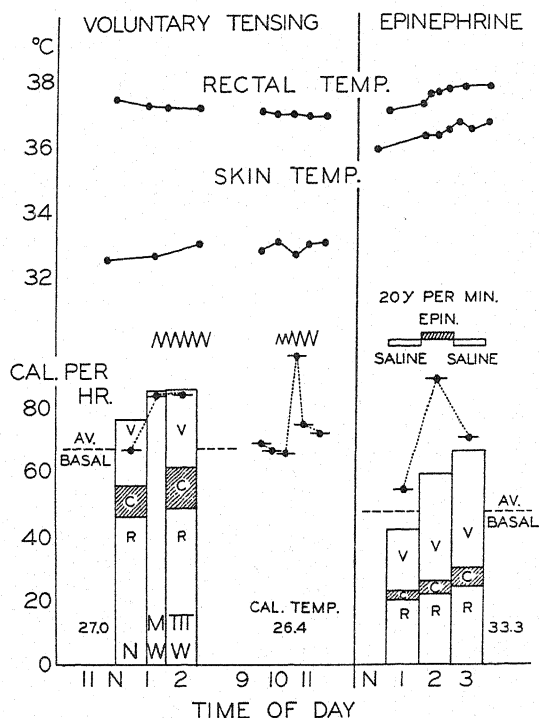


Fig. 14 The first two experiments show the effect of voluntary tensing of the muscles on the heat production. In the first experiment there was vigorous tensing in the second and third periods. In the second experiment the first two short periods were basal, in the third the tensing was mild, in the 4th it was vigorous. The third experiment illustrates the action of epinephrine.

most sensitive type of instrumental detection needle electrodes as used by Burton and Bronk ('37) in their preliminary report on cats showed twitches of single muscle units. Myographic tracings have been made by several investigators. Hemingway and Hathaway ('41) made a careful study of three well-trained dogs with electrodes over the muscles and

with the hind quarters on a sensitive balance. Their basal metabolism rates during the comfortable control periods averaged 34 cal. per square meter per hour. The temperature was then dropped 5°C.-6°C., the metabolism rose 5 to 10% (average 7) before shivering and 24-42% during shivering. The electrodes picked up the onset of shivering very definitely. These authors conclude that the chemical regulation without any sign of shivering is only 7% on the average and is so small that it is without practical significance in protecting against exposure to cold.

An experiment at Woods Hole was performed on one of us through the courtesy of Dr. John F. Perkins, Jr. Sensitive electrodes on 6 parts of the body showed almost no change during mild tensing before a chill but vigorous response at the onset of real or simulated chill.

One attempt to evaluate the effect of maximal tensing by Swift ('32) showed an increase of 36% in metabolism. Another attempt made by one of us has been described by Hardy, Milhorat and DuBois ('38) and the results are shown in figure 14. After one basal period the man started tensing his muscles as hard as he could, first in one extremity and then in another, and kept this up for 50 minutes. At the end he was very tired but he had increased his heat production only 25% and his elimination 11%. In 1950 more tests were made using a Benedict-Sanborn apparatus with 6-minute periods. The results are shown in the middle of figure 14. The first two periods were basal. In the third period he imitated every 15 seconds the slight muscular tensings that precede a chill using first one arm, then the other and then each leg. In spite of this the level of the metabolism was practically unchanged. In the 4th period he exerted severe tensing for 5 seconds, relaxing 5 seconds, and kept this up for 10 minutes. The metabolism rose 42% and then fell rapidly after he stopped. In figure 14 is also shown the sharp rise in metabolism of a normal man in the calorimeter when a small amount of epinephrine was added to

his intravenous saline infusion. The stimulating effect of epinephrine was studied extensively by Sandiford ('20).

The effects of tension of muscles and rigidity have not received the attention they deserve. Roaf in 1912 and 1913 abolished the decerebrate rigidity of cats by means of curare and by cutting the nerves to all 4 limbs. There was no significant reduction in the carbon dioxide output and only a slight fall in the oxygen consumption. Dusser de Barenne and Burger ('24) studied cats soon after decerebration and then "abolished rigidity" by cutting the sciatic and femoral nerves and the brachial plexus. The drop in metabolism was small.

Grafe ('20-'23) discussed muscle tone and total metabolism, reviewed the literature and cited his own experiments on cataleptic spasm in hypnosis and spastic paralysis. He concluded that a tension of muscles does not lead to an increase in oxidation, as long as there is no significant work performed.

Statistical analysis

The statistical analysis of the results has been difficult and many methods have been explored.³ It happens that the simplest method has given the most satisfactory results. It is apparent from figures 1 and 2 that there is a distinct tendency for a metabolism higher in the cold than in the comfort zone. The probability that this is not due to chance was determined with the aid of table 2. There were 10 cases where the metabolism was measured both in the comfort and in the cold zones (H. G., P. N., A. S., 1939; A. S., 1940; H. E., B. R., G. C., P. H., L. T., and R. C.). It is necessary to list A. S. as two different women since her weight was 76 kg in 1939 and 64 kg in 1940.

In each case the woman's basal in the comfort zone was used as a standard for comparison. In 4 cases it was determined from the average of the first and second hours of

³ We are greatly indebted to Dr. Donald R. Charles of Rochester University, Rochester, New York, for his help in this analysis

one calorimeter experiment, in 6 cases from the average of the first and second hours of two or three experiments. In table 2 are tabulated the number of times the tests in the cold zone were higher (+) or lower (—) than the standard for that woman in the comfort zone. It will be noted that in the "cold zone" (22°C.-26.9°C.) out of a total of 32 experiments the metabolism was higher 23 times and lower 9

TABLE 2
Cold zone deviations from each woman's average in comfort zone

CAL./SQ. M PER HOUR	"COLD ZONE" 22°C.-26.9°C.		ZONE 22-24.9	
	+	—	+	—
0-0.9	3	4	2	2
1-1.9	6	3	6	
2-2.9	2	1	2	1
3-3.9	3	1	2	1
4-4.9	1			
5-5.9	3		3	
6-6.9	4		2	
7-7.9	1		1	
No.	23	9	18	4

TABLE 3
Ten women's basals in comfort zone

CAL./SQ. M PER HOUR	DEVIATIONS FROM AV. 30.7	
	+	—
0-0.9	1	4
1-1.9	1	
2-2.9	1	1
3-3.9	1	1

times. The probability (P) that this would occur by chance is 0.01. Five of the 9 minus figures came between 26°C.-26.9°C. which is the edge of the comfort zone. If we consider the 22 tests between 22 and 24.9 there are 18 above the comfort zone standard, and only 4 below; $P = 0.002$. The average rise in metabolism in the zone below 22°C.-24.9°C. was 7.6% (s.d. 7.5%; standard error of the arithmetic mean 1.6%). Table 3 shows the rather small deviations of the

comfort zone standards of these 10 women from their average of 30.7 cal. per square meter per hour.

At present the evidence is that the slight muscle contractions reported as tensing by our subjects have so small an effect on metabolism that they cannot account for all of the rise found in some of the women in the cold zone. A small increase in the release of epinephrine or some similar substance could account for the rise but there is little evidence for or against epinephrine effect. There is no evidence that the increase is due to anything resembling the specific dynamic action of foods. The nitrogen excretion of the women remained at a low level; the respiratory quotients were very uniform. We are left, therefore, with the presumption that in some but not all women there is a true chemical regulation in the old Rubner sense but we must agree with Hemingway and Hathaway ('41) in their conclusion that it is so small that it has little practical significance in protecting against severe exposure to cold but might be helpful in mild exposure.

Van't Hoff effect on basal metabolism

Recently several investigators have discussed the question of making allowances for differences in body temperature when calculating the basal metabolic rate. A good many years ago it was shown (DuBois, '21) that in fever the metabolism is raised about 13% for each degree Centigrade rise in rectal temperature. Benedict, Benedict and DuBois ('25) pointed out the probable increase of metabolism in peripheral tissues warmed in hot air baths. What we need to know in our experiments on women is the temperature of the peripheral tissues in cold environments, the core temperature and the average body temperature.

Figure 11 shows the rectal and average skin temperatures, and from them the average body temperature can be approximated by using the formulas of Hardy, Milhorat and DuBois ('41). At all temperatures the rectal temperature is assigned the greatest weight and the skin temperature, rep-

representing the peripheral tissues, weights depending on the depth of cold penetration. The skin temperature is weighted as follows: Environments 22°C.-25°C. 40%, 25°C.-28°C. 30%, 28°C.-33°C. 20% and 33°C.-36°C. 10%. This allows one to make a curve of "average body temperature" from 33.8°C. at 22, to 37.1°C. at 35. In the cold region 22-25 the average body temperature is 34.1°C., in the comfort zone 36.3°C. and in the warm zone 37.0°C.

Since in the cold region the average is 2.2°C. lower than in the comfort zone we might expect 2.2×13 or 28.6% lowering of the standard basal metabolism, and in the warm zone 0.7×13 or 9.1% rise. The fact that the metabolism in the men and some of the women does not change significantly with environment shows that there must be a chemical regulation which increases metabolism in the core of the body to make up for the lowering in the periphery.

The calculations will never be exact because we do not know the percentage of metabolism that is derived from the peripheral tissues. There is reason to believe that the viscera in the core of the body have a much higher rate per kilogram than the skin, subcutaneous tissue and resting muscles of the periphery. If we assume only half the values of the previous calculation this would indicate a chemical regulation by the internal organs which amounts to an increase of 14% of the total metabolism in the cold before any signs of shivering.

*Comparison of the mechanics of temperature
regulation in men and women*

A summary of the differences between the sexes in the mechanics of heat loss is presented in figures 15 and 16. This is only a small segment of the many differences between men and women for which we are all grateful.

Between the calorimeter environments of 24°C. and 33°C. there are enough experiments on women to give satisfactory statistics. The two extremes of the lines are based on fewer subjects. Unfortunately there are only two men available for

comparison under exactly similar conditions. Both had been found close to the average normal in many kinds of metabolic experiments. J. D. H. at the age of 33 in the calorimeter series, had an average basal rate 12% below the Boothby, Berkson and Dunn ('36) "standards." E. F. D. B. at the average age of 55 was 7% below the standards. These are both

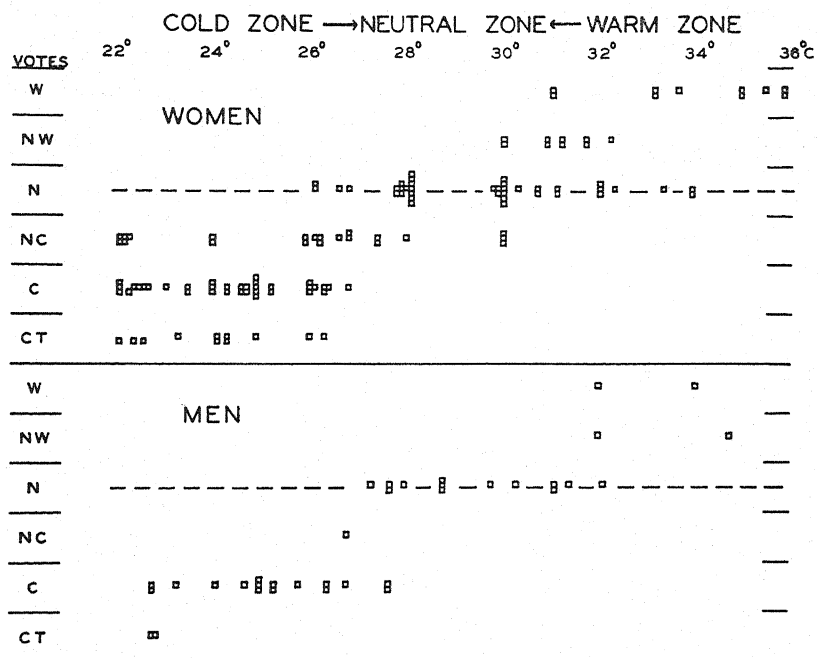


Fig. 15 Comfort votes of 13 women and two men in the environmental temperatures from 22° to 36°C. The votes for both the first and second hours are recorded except in those cases where only one basal hour was available. W, too warm; N. W., neutral to warm; N, neutral (comfortable); N. C., neutral to cold; C, too cold; C. T., cold with tensing of a few muscles.

very close to the average now found with modern basal technique in physiological studies.

The comfort votes of the men and women are shown in figure 15 and, in general, the differences appear small. The women seem to have a somewhat wider comfort zone. Between 28 and 31 all of the women and men were comfortable

or nearly so. In this zone the average skin temperature of the women was 33.9°C .

Figure 16 compares the smoothed curves for the 13 women and the two men. The figures are taken from the first experimental hours only except in the case of J. D. H. whose basal

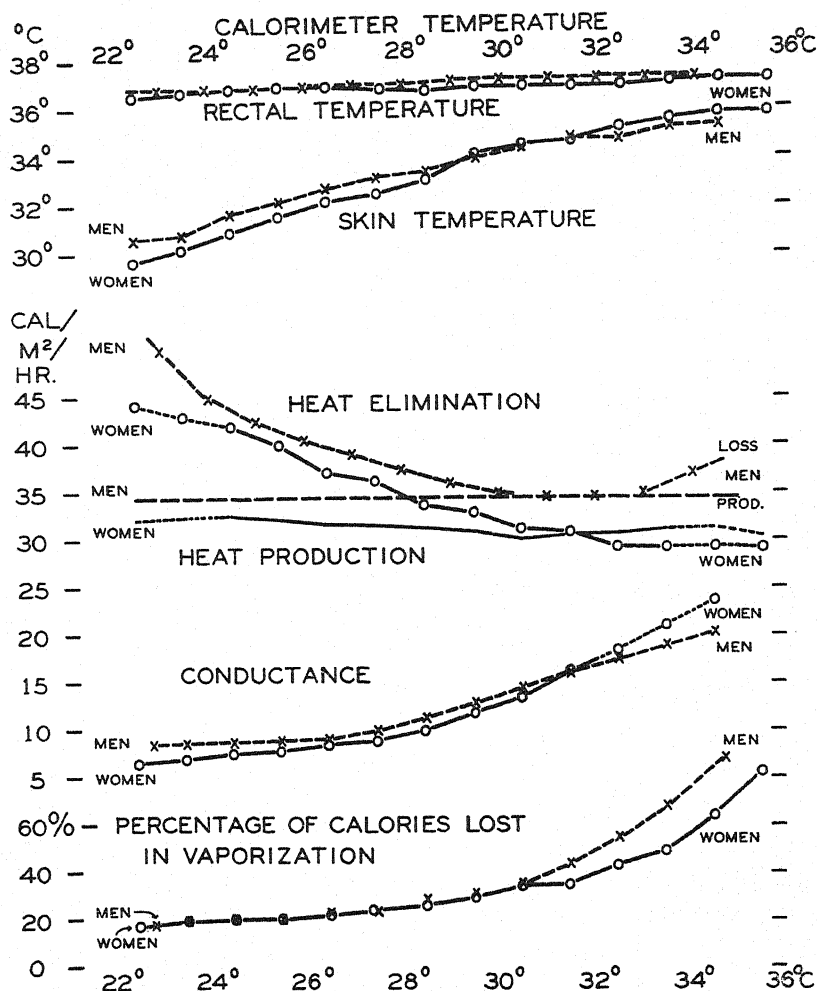


Fig. 16 Comparison of smoothed curves of data for 13 women and two men. Only the first experimental hours are used. The dashed line shows the heat production of the men which was level throughout the temperature range.

metabolism was calculated from the average of the first two hours in the few experiments when he slept part of the first hour.

The rectal temperatures at the top of the chart are practically identical, both only 0.3°C . lower in the cold than in the comfort zone. The skin temperatures of the women were about a degree lower than those of the men in the cold zone and half a degree higher in the warm zone. The heat production of the men was level throughout the range at 34.5 per square meter per hour. In the cold zone the average for the women was about 7% lower than the men, in the comfort zone about 12% lower. There was, however, a wide scatter. In the 42 separate hours in which women were studied between 22.9 and 25.2, 6 showed metabolic rates above the range 32.8-36.1 (cal. per square meter per hour) found in the men. Eleven were within this range and 25 of the hours were below the men's range.

Heat elimination of women was distinctly lower than that of men especially at the two extremes of the curve and the loss of heat from the bodies of the subjects in the cold zone (negative storage, heat loss minus heat production) was less for the women than for the men, especially during the second hours. Even so, in the women it averaged 10 cal. per square meter per hour in the zone from 22-24.9 in the first hour, and 18 for the first two hours.

Conductance (conductivity) of heat through the peripheral tissues was calculated by dividing the heat elimination (cal. per square meter per hour) by the difference between rectal and skin temperatures. It was lower for the women in the cold and comfort zones showing a greater resistance to the loss of heat. In the warm zone it was higher and they lost heat more easily. One would assume that the lower conductance of women was due to a thicker layer of subcutaneous fat. This may not be the correct explanation. The young woman A. S. at temperatures between 22 and 23 in 1939, when she weighed 76 kg, had an average conductance of 7.45. In

1940 after she had lost 12 kg it was 5.65. The reason for this unexpected change is not clear.

The percentage of calories lost in vaporization was identical until the environmental temperature was over 31. Then the women did not need to sweat as much as the men.

Other differences between men and women were shown in the approach to chills. The women were able to stand exposures to the cold much longer than the men before they started to shiver. There were 8 experiments with chills in women, 6 with men. Just before shivering the average rectal temperatures were identical, 36.7°C., but the women had tolerated an average skin temperature 0.3° lower than the men.

A summary of all these rather small differences adds up to the physiological advantage of the women in both the cold and warm zones. In the cold zone most of the women can increase their heat production without muscular movement. Their skin temperatures are lower and they lose less heat through the skin and subcutaneous tissues. They have a wider comfort zone. In warm environments their metabolism is so much lower than that of the men that they do not need to lose as much heat per unit of surface and, therefore, do not need to sweat as much.

In general this confirms the conclusions reached in our previous report (Hardy, Milhorat and DuBois, '41) but the differences are less marked. This is due to the inclusion of 7 more women, and slightly different methods of calculation.

SUMMARY AND CONCLUSIONS

Seventy-six experiments in the Sage respiration calorimeter were made on 13 normal young women at environmental temperatures between 22°C. and 35°C. The measurements in hourly periods included heat production, respiration quotient, rectal temperature, average surface temperature, total heat loss, conductance and the percentages of heat lost by

radiation, convection and vaporization. Careful note was made of muscle tensing, shivering and sensations of cold, warmth and comfort.

The average basal metabolism of the 13 women was 31.2 cal. per square meter per hour. This is so much lower than the heat production of men per unit of surface that women have an advantage over men in the warm zones and do not need to sweat as much. In the cold zone some, but not all, women lose heat less rapidly than men.

In the cold zone the women lost about 67% of their heat by radiation and this percentage dropped in linear fashion to zero when the calorimeter temperature was the same as skin temperature. Vaporization accounted for only 16-18% of the total loss at the coldest temperatures and reached the conventional figures of 24-25% only at environments of 27°C.-28°C. It is obvious that fixed percentage figures for radiation, convection and vaporization apply to only one strictly specified set of conditions.

The comfort or neutral zone for women, naked, and in strict basal conditions, extended from about 26°C. to 30.9°C. In this zone the average skin temperature was 33.9°C. and in the upper part of the zone heat production closely approximated heat elimination. Of the 10 women studied at temperatures of 28 and below, 6 showed an increase in metabolism in the cold greater than the amount that could be ascribed to muscle tension or restlessness. This is evidence that some but not all women are subject to Rubner's "chemical regulation" of metabolism but the amount involved is small.

In the cold zone the peripheral tissues lost so much heat that the average temperature of the body dropped 2.8°C. before the onset of shivering. The metabolism of the peripheral tissues falls with lower temperatures. Since the total metabolism remains constant or rises there must be an increase in the metabolism of the core of the body which compensates for the decrease in the periphery. In this sense there is a chemical regulation in men and women.

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OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1953 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1953.

Chairman, Nominating Committee:

DR. PHILIP HANDLER
*Department of Biochemistry and Nutrition
Duke University School of Medicine
Durham, North Carolina*

BORDEN AWARD IN 'NUTRITION

Nominations are solicited for the 1953 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it will be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1953. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1953. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

Chairman, Nominating Committee:

DR. LEO T. SAMUELS
*Department of Biological Chemistry
University of Utah Medical School
Salt Lake City, Utah*

THIAMINE METABOLISM OF WOMEN ON CONTROLLED DIETS

I. DAILY URINARY THIAMINE EXCRETION AND ITS RELATION TO CREATININE EXCRETION^{1, 2}

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AND CLARA A. STORVICK

WITH THE TECHNICAL ASSISTANCE OF KATHERINE DING AND BETTY J. GRAVES
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ONE FIGURE

(Received for publication April 21, 1952)

The determination of urinary thiamine excretion has been used in many laboratories in assessing the thiamine nutrition of man since the picture it gives is fairly reliable. Mickelsen, Caster and Keys ('47) stated that "thiamine excretion values appear in a general way to be linearly related to the thiamine intake." Alexander, Landwehr and Mitchell ('46) reported that "the excretion of thiamine into the urine by normal subjects is directly related to the amount of the vitamin administered."

Adamson et al. ('45) indicated that the excretion of thiamine is related to the excretion of creatinine, and that this ratio is fairly constant from voiding to voiding in a given

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² This study was made possible by grants from the Williams-Waterman Fund of the Research Corporation of New York City.

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individual. If this is true, the amount of thiamine, in micrograms per gram of creatinine, found in a single voiding of urine would indicate the thiamine nutrition of the individual. The use of this method would mean a great saving of time to the analyst. No further information concerning this method of estimating thiamine nutrition has been found in the literature.

This paper reports data on the daily urinary excretion of thiamine and creatinine of subjects on a controlled intake. The nutritional status of the subjects with respect to thiamine was evaluated on the bases of (1) total daily excretion of thiamine, (2) percentage of thiamine intake excreted, and (3) the ratio of thiamine to creatinine in the urine, expressed as micrograms of thiamine per gram of creatinine.

The per cent excretion of a 5-mg oral test dose of thiamine hydrochloride was determined for some of the subjects. In addition, a very brief study was conducted to assess the validity of estimating the status of thiamine nutrition of an individual from the thiamine to creatinine ratio in single voidings. Determinations were also made on two subjects on controlled diets but ingesting therapeutic amounts of thiamine.

EXPERIMENTAL

This work formed a part of a larger investigation conducted during the years 1949, 1950 and 1951. The purpose of the entire experiment was to investigate the effect of two different levels of thiamine intake on the thiamine nutrition of apparently normal women on a controlled diet, as shown by determinations of urinary excretion of thiamine and creatinine, and the concentration of thiamine in the blood.

Each year, the study was divided into two periods: (1) when the daily thiamine intake was maintained at 500 μ g per 1,000 Cal., by the addition of 400 μ g of thiamine hydrochloride in aqueous solution to the thiamine provided by the diet, and (2) when the daily thiamine intake was maintained at 300 μ g per 1,000 Cal., the amount provided by the diet. The

duration of periods 1 and 2 was, respectively, 31 and 21 days in 1949, 19 and 15 days in 1950 and 15 and 14 days in 1951.

Diet and supplement

The diet used was a modification of the diet of Giffit and Hauck ('46). It provided approximately 70 gm of protein, 80 gm of fat and 260 gm of carbohydrate, to give a total daily intake of approximately 2,000 Cal. Food tables⁵ were used to evaluate these constituents. Representative samples were analyzed for thiamine by a modification of the method of Hennessy and Cerecedo ('39), and it was found that approximately 600 μ g were provided by this diet. The foods were weighed and the quantity of each consumed daily remained constant throughout the experimental periods. During the 1949 study, the diet was planned to provide only 25 mg of ascorbic acid per day, in order that a special concurrent study could be made of the levels of ascorbic acid in blood and urine (Davey et al., '52). During the 1950 and 1951 studies, the riboflavin content of the diet was maintained at 1.2 mg per day. With these exceptions, the diet was adequate with respect to all other known nutrients.

Subjects

There were 4 subjects for the 1949 study, three subjects for the 1950 study, and 4 subjects for the 1951 study. M.L.W. served as a subject during all three studies and C.A.S. served as a subject during the 1949 and 1951 studies. The subjects were women graduate students and one staff member, and ranged in age from 24 to 44 years. Three of them (M.L.W., H.H.Y. and K.D.) are Chinese and S.W.W. is Korean.

Methods

Urinary thiamine was determined by a modification of the thiochrome method of Hennessy and Cerecedo ('39). The

⁵ "Composition of Foods, Raw, Processed, Prepared" by the Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, Agriculture Handbook 8, 1950.

suggestion of Mickelsen, Condiff and Keys ('45) of adjusting the pH just before extracting the thiochrome was followed. Urinary creatinine was determined by Folin's method as described by Hawk, Oser and Summerson ('47).

RESULTS AND DISCUSSION

The first 5 days of each period of controlled thiamine intake were considered as an adjustment time, and the values for urinary thiamine and creatinine for those days were omitted in calculating the data shown in table 1.

The results were evaluated on the following bases suggested as evidences of adequate thiamine nutrition: (1) a mean daily urinary thiamine excretion of 100 μ g (Mason and Williams, '42); (2) a mean daily urinary excretion of 13% of the thiamine consumed (Giff and Hauck, '46; Melnick, Field and Robinson, '39); and (3) a mean daily urinary excretion of 150 μ g of thiamine per gram of creatinine excreted (Adamson et al., '45).

(1) On the basis of a mean daily thiamine excretion of 100 μ g, an intake of 500 μ g per 1,000 Cal. was judged to be adequate for all of the subjects. An intake of 300 μ g per 1,000 Cal. was judged to be adequate for only one of the subjects (H.H.Y.). Although M.L.W. excreted over 100 μ g per day in period 2, 1949, she did not do so in the two subsequent studies. Therefore, an intake of 300 μ g per 1,000 Cal. was judged to be inadequate for her. Variation in day-to-day excretion was greater for all subjects during period 1 (fig. 1).

(2) On the basis of a mean daily urinary thiamine excretion of 13% or more of the intake as a criterion of good nutrition, an intake of 500 μ g per 1,000 Cal. was judged to be sufficient for all but one subject (H.A.L.). This subject had an average 24-hour excretion of just 100 μ g. An intake of 300 μ g per 1,000 Cal. was judged to be adequate for two subjects (B.L.D. and H.H.Y.). Again, M.L.W. excreted more than the critical percentage of her intake in 1949, but not in 1950 and 1951.

(3) On the basis of the urinary excretion of 150 μg of thiamine per gram of creatinine as the critical ratio, the higher thiamine intake was judged to be adequate for all but two subjects (H.A.L. and C.A.S.). The lower thiamine intake was judged to be adequate for none of the subjects. H.A.L. and C.A.S. excreted less than 50 μg of thiamine per gram of creatinine during period 2. According to Adamson et al.

TABLE 1

The urinary excretion of thiamine and creatinine of women on controlled diets

YEAR	SUBJECT	DAILY THIAMINE INTAKE	AVERAGE URINARY EXCRETION			
			Thiamine	Thiamine	Creatinine	Thiamine/ Creatinine
		$\mu\text{g}/1,000 \text{ Cal.}$	$\mu\text{g}/24 \text{ hr.}$	% of intake	$\text{gm}/24 \text{ hr.}$	$\mu\text{g}/\text{gm}$
1949	M.L.W.	495	227	22.7	1.10	206
		299	107	17.8	1.08	99
	B.L.D.	495	229	22.9	0.83	276
		299	84	14.0	0.80	105
	C.A.S.	495	142	14.2	1.42	100
		299	48	8.0	1.37	35
1950	H.H.Y.	495	246	24.6	1.05	234
		299	109	18.2	1.02	107
	M.L.W.	484	163	16.3	1.07	152
		288	50	8.3	1.07	47
	K.D.	484	184	18.4	1.09	169
		288	57	9.5	1.08	53
1951	S.W.W.	484	159	15.9	0.84	189
		288	61	10.2	0.89	69
	M.L.W.	504	268	26.8	1.15	233
		308	77	12.8	1.11	69
	H.A.L.	504	100	10.0	1.25	80
		308	34	5.7	1.30	26
1949	C.A.S.	504	133	13.3	1.48	90
		308	47	7.8	1.56	30
	R.B.D.	504	276	27.6	1.10	250
		308	70	11.7	1.09	64
	B.W.C. ^{1,2}	1548	806	27.1	1.05	768
		1339	549	21.4	1.01	544
1950	H.H.Y. ^{1,3}	2327	1078	17.9	1.07	1007
		2172	1000	17.8	1.08	926

¹ These subjects were ingesting therapeutic amounts of a vitamin preparation containing thiamine.

² B.W.C. was under a doctor's supervision for a nephritic condition.

³ This subject received 2,600 Cal. daily.

DAILY THIAMINE EXCRETION BY ADULTS RECEIVING CONTROLLED
DIETS WITH TWO LEVELS OF THIAMINE INTAKE

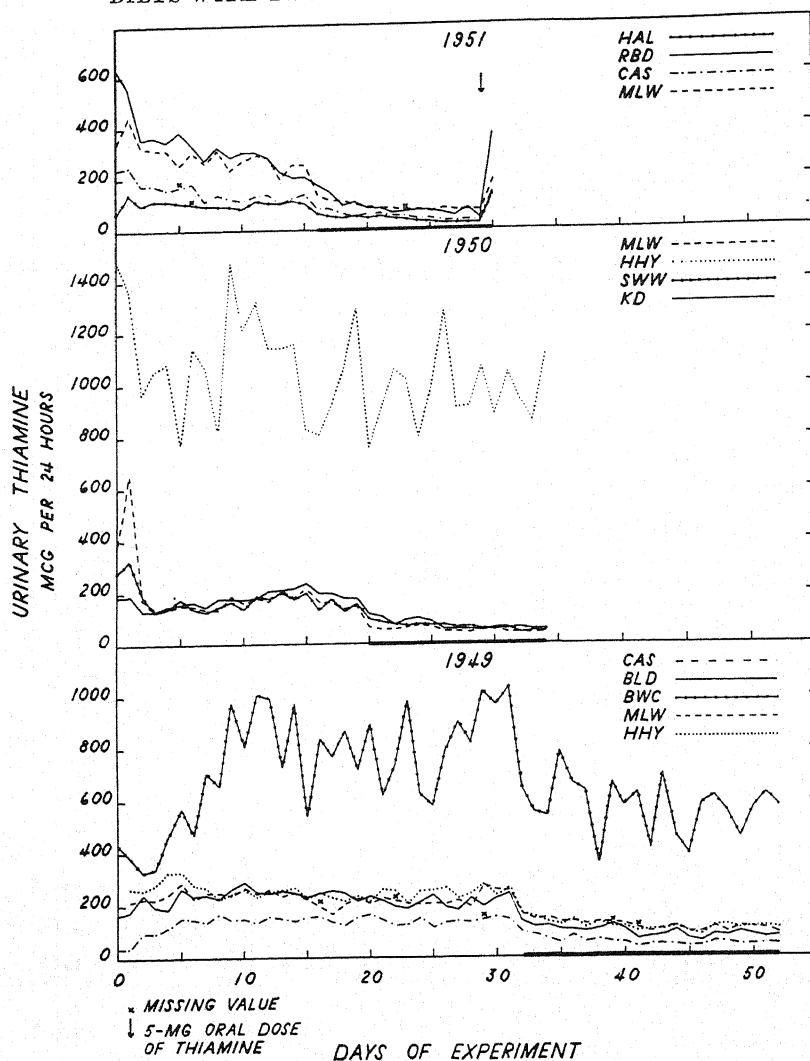


Fig. 1 The periods of lowered thiamine intake are indicated by the heavier base lines.

('45), an excretion of about 50 μ g of thiamine per gram of creatinine suggests subnormal thiamine nutrition. Evaluation of thiamine nutritional status by this means gives results which agree generally with those obtained by the other means.

By all three methods of evaluation, it appears that a daily thiamine intake of 500 μ g per 1,000 Cal. was sufficient for 6 of the 8 subjects but probably borderline for the other two. This would suggest that the recommended daily allowance of the National Research Council ('48) does not provide a very large safety factor in all instances. A daily intake of 300 μ g per 1,000 Cal. appeared, by all three methods of evaluation, to be inadequate for 6 subjects and borderline for the other two.

The fact that there is wide variation in the daily excretion of thiamine when the intake level is high (fig. 1) is not of clinical importance, since even the lower excretions during period 1 would have yielded the same conclusions regarding thiamine nutrition as did the average values. Since the daily fluctuations are much smaller when the excretion is less, the chances of mis-evaluating a thiamine-deficient person on the basis of one day's excretion are also less.

ISOLATED OBSERVATIONS

Five-milligram oral test dose

At the end of the 1951 study, the 4 subjects ingested orally a 5-mg test dose of thiamine hydrochloride, and the 24-hour excretion of the test dose was determined. All of the subjects excreted less than 7% of the dose (M.L.W., 2.4%; H.A.L., 2.1%; C.A.S., 2.2%; and R.B.D., 6.6%) which, according to Melnick, Field and Robinson ('39) and substantiated by Robinson, Melnick and Field ('40) and Giff and Hauck ('46), is indicative of deficient thiamine stores. This is further evidence that the 300 gm of thiamine per 1,000 Cal. intake of period 2 was not sufficient for these subjects. R.B.D. had taken a liver and vitamin preparation prior to the study, and her excretion of a higher percentage of the test dose was

probably an indication that she had maintained greater thiamine stores than the other subjects.

Ratio of thiamine to creatinine in separate voidings

After the close of the 1951 study, each subject collected and preserved separately each voiding for two days. A summary of the results is given in table 2. It can be seen that there is variation of the thiamine to creatinine ratio in separate voidings, particularly when the ratio was high. However, there were only two of the 34 instances which were very far

TABLE 2
*Ratio of thiamine to creatinine in separate voidings
for 4 subjects in two 24-hour periods*

SUBJECT	RATIO OF THIAMINE TO CREATININE			
	First day		Second day	
	Range in separate voidings	24-hour value	Range in separate voidings	24-hour value
	$\mu\text{g/gm}$	$\mu\text{g/gm}$	$\mu\text{g/gm}$	$\mu\text{g/gm}$
M.L.W.	276-371	314	367-621	431
H.A.L.	101-108	105	132-157	146
C.A.S.	144-250	184	167-209	180
R.B.D.	183-508	249	149-224	180

from the 24-hour ratios. The use of the urinary thiamine to creatinine ratio in single voidings as a means of evaluation of thiamine nutrition would seem to be warranted, particularly under survey conditions, since this ratio was, for practical purposes, the same as the 24-hour ratio, and the evaluation of thiamine nutrition by using the 24-hour ratio led to the same conclusions as evaluation by other methods.

*Values for two subjects who were receiving
thiamine supplements*

These subjects followed controlled but modified diets. The thiamine intakes and the values found are given in table 1

(B.W.C., 1949, and H.H.Y., 1950). As would be expected, the urinary excretion of thiamine of these subjects was greater than that of the normal subjects throughout periods 1 and 2.

SUMMARY

When 8 normal women were maintained on an intake of 500 μg of thiamine per 1,000 Cal. the average daily urinary excretions of thiamine ranged from 100 to 276 μg . When the thiamine intake was reduced to 300 μg of thiamine per 1,000 Cal. the average daily urinary excretions of thiamine ranged from 34 to 109 μg . The thiamine excretion expressed as per cent of thiamine intake ranged from 10.0 to 27.6 for the higher intake period and from 5.7 to 18.2 for the lower intake period. Urinary thiamine excretions expressed in terms of micrograms of thiamine per gram of urinary creatinine ranged from 80 to 276 for the first period, and from 26 to 107 for the second period of intake.

Using an excretion of 100 μg of thiamine per 24 hours, an excretion of 13% of the daily thiamine intake and an excretion of 150 μg of thiamine per gram of creatinine as indications of good nutrition with respect to thiamine, an intake of 500 μg of thiamine per 1,000 Cal. was judged to be adequate for 6 of the 8 subjects and borderline for the other two. An intake of 300 μg of thiamine per 1,000 Cal was inadequate for 6 of the subjects and borderline for the other two.

The response of 4 subjects to a 5-mg oral test dose of thiamine hydrochloride given on the last day of the period of lowered thiamine intake indicated that the tissues of all the subjects were low in thiamine.

The determination of thiamine in micrograms per gram of creatinine in individual voidings seemed to indicate that the ratio of thiamine to creatinine is fairly constant and, except in two of 34 instances, could have been used as well as the 24-hour ratio for a rough estimation of the status of thiamine nutrition of the subjects.

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THIAMINE METABOLISM OF WOMEN ON CONTROLLED DIETS

II. DAILY BLOOD THIAMINE VALUES ^{1, 2}

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ONE FIGURE

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No report has been found in the literature in which daily fasting blood thiamine values have been determined for subjects whose thiamine and caloric intakes have been known and controlled for extended periods. It was felt desirable to know how greatly the levels of thiamine in the blood fluctuate under controlled conditions. This paper is a report of the values obtained for thiamine in whole blood and of calculated values of thiamine in packed blood cells. In addition, the experiment was planned to determine whether any change in blood thiamine concentration would occur when the thiamine intake was reduced from 500 μg to 300 μg per 1,000 Cal.

Some determinations of blood thiamine were also made on two subjects on controlled diets but ingesting therapeutic

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amounts of thiamine, on two ambulatory patients receiving thiamine therapy, and on 5 normal people having self-selected diets.

EXPERIMENTAL

The determination of blood thiamine formed a part of a more extensive investigation conducted during the years 1949, 1950 and 1951. There were 4 subjects in the 1949 and 1951 studies and three in 1950. A detailed description of the subjects, of the diet which afforded approximately 2,000 Cal. daily, and of the plan of the experiment has been given in the report by Louhi, Yü, Hawthorne and Storvick ('52).

Collection of blood samples

Every morning before breakfast, free-flowing blood from a finger puncture was collected in a heparinized ⁵ 3 × 100 mm capillary tube for hematocrit determination and in a small vial for thiamine determination. Aliquots of blood for the latter were measured immediately so that no anticoagulant was needed.

Method of analysis

Whole blood thiamine was determined by a modification of the microthiochrome method of Burch et al. ('52a). Results were calculated by reference to a standard curve based on the recovery of various amounts of thiamine hydrochloride added to whole blood. Thiamine values were calculated for packed cells from the whole blood values and hematocrit readings, assuming that 10% of the whole blood thiamine is contained in the plasma. This assumption may not hold in every instance. According to some preliminary studies made in this laboratory, the plasma contained between 14 and 15% of the total blood thiamine. However, for the purpose of comparison with Burch et al. ('50) it was assumed that the plasma contained 10% of the total thiamine in the blood.

⁵ Heparin was obtained through the courtesy of Roche-Organon, Nutley, New Jersey.

The results of analyses performed during the first 10 days of the 1949 study were not considered valid because of loss of activity in the enzyme preparation. Consequently, the remainder of the samples obtained in 1949, and all of those obtained in 1950, were deproteinized, frozen in dry ice, and stored in a deep freeze until analysis in 1951 when a satisfactory and stable concentrate of acid phosphatase had been prepared. The samples from the 1951 study were analyzed immediately upon collection. The activity of the enzyme preparation was tested using p-nitrophenyl phosphate and cocarboxylase⁶ as substrates. It was observed that fluorometric readings of samples which had been frozen were higher than those of aliquots of the same sample analyzed immediately upon collection. For this reason, the samples frozen for one and two years were evaluated using a standard curve based on the recovery of various amounts of thiamine hydrochloride added to whole blood, which was then deproteinized and frozen before being analyzed.

RESULTS

The daily values for micrograms of thiamine per 100 ml of whole blood are shown in figure 1. It can be seen that there were individual differences and considerable variation from day to day for a given individual during either period 1 when the thiamine intake was 500 μ g per 1,000 Cal. or period 2 when the intake was 300 μ g per 1,000 Cal. It can also be seen that the lowering of the thiamine intake was not reflected in a proportional lowering of the blood thiamine values.

The average of 204 determinations made during all of the periods when the daily thiamine intake was 500 μ g/1,000 Cal. (called "periods 1"), was 3.75 μ g per 100 ml of whole blood; the average of 193 determinations when the daily thiamine intake was approximately 300 μ g/1,000 Cal., made during all of the periods (called "periods 2"), was 3.40 μ g. The overall

⁶ Cocarboxylase was obtained through the courtesy of Merck and Company, Rahway, New Jersey.

range was from 2.1 to 6.1. The individual average values are given in table 1.

R.B.D., prior to the 1951 study, had taken a liver and vitamin preparation, and her blood thiamine values were

Daily blood thiamine in adults receiving controlled diets with two levels of thiamine intake

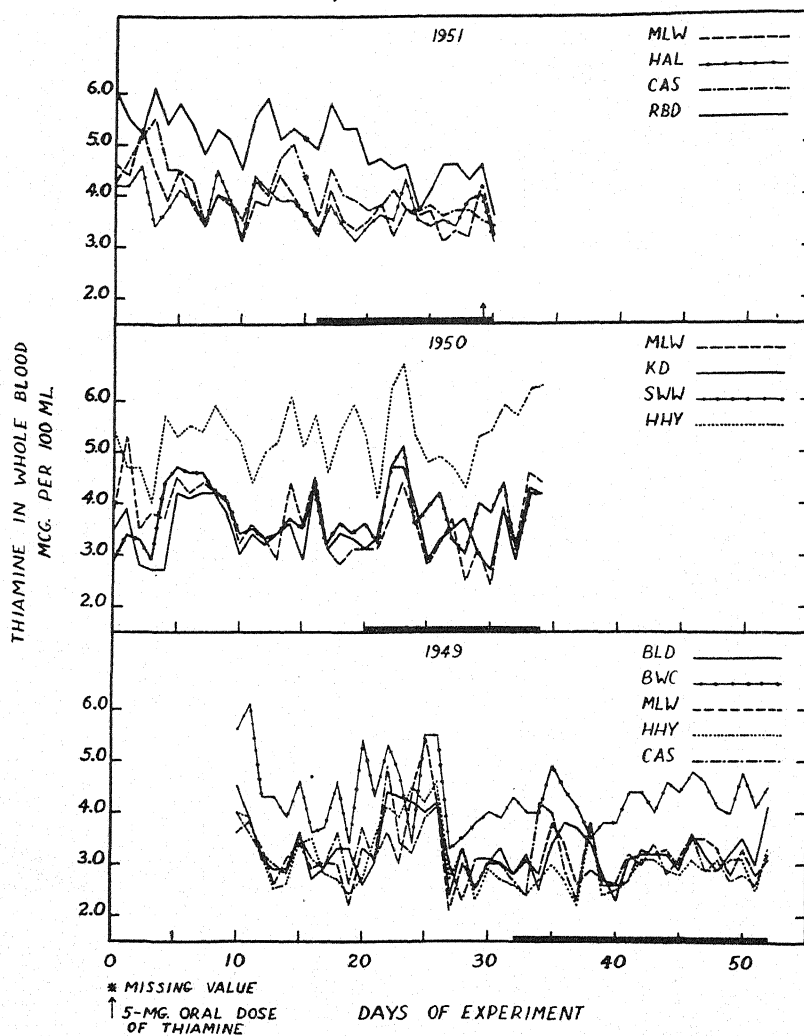


Fig. 1 The periods of lowered thiamine intake are indicated by the heavier base lines.

consistently higher throughout the experimental period (fig. 1 and table 1) although her excretion of thiamine was similar to that of the other subjects (Louhi et al., '52).

Analysis of variance indicated that variation in blood thiamine due to change in thiamine intake was significant in 1951 but not significant in 1949 and 1950.

TABLE 1
Blood thiamine levels of women on controlled diets

YEAR	SUBJECT	DAILY THIAMINE INTAKE	AVERAGE BLOOD THIAMINE LEVEL	
		$\mu\text{g}/1,000 \text{ Cal.}^1$	$\mu\text{g}/100 \text{ ml blood}$	$\mu\text{g}/100 \text{ ml packed cells}$
1949	M.L.W.	495	3.3 ²	7.3 ²
		299	3.1	6.8
	B.L.D.	495	3.4 ²	8.6 ²
		299	3.2	7.9
	C.A.S.	495	3.3 ²	7.0 ²
		299	3.0	6.3
	H.H.Y.	495	3.3 ²	8.1 ²
		299	2.8	6.6
1950	M.L.W.	484	3.8	8.2
		288	3.4	7.6
	K. D.	484	3.5	8.1
		288	3.6	8.6
	S.W.W.	484	3.7	8.5
		288	3.9	8.7
	M.L.W.	504	4.1	8.9
		308	3.5	7.5
1951	H.A.L.	504	4.0	9.2
		308	3.6	8.1
	C.A.S.	504	4.4	9.0
		308	3.8	7.6
	R.B.D.	504	5.4	11.2
		308	4.6	10.0
	B.W.C. ³	1548	4.4 ²	9.4 ²
		1339	4.3	9.3
1950	H.H.Y. ^{3, 4}	2327	5.2	12.0
		2172	5.4	12.5

¹ Daily intake: 2,000 Cal.

² Blood thiamine data for the first 10 days were not included.

³ These subjects were ingesting therapeutic amounts of a vitamin preparation containing thiamine.

⁴ This subject received 2,600 Cal. daily.

The average of 188 determinations made during all the "periods 1" was 8.49 μg of thiamine per 100 ml of packed cells, and the average of 186 determinations made during all the "periods 2" was 7.69. The overall range was from 4.7 to 13.0 μg per 100 ml of packed cells. The individual average values are shown in table 1.

Isolated observations

Values for two subjects who were receiving thiamine supplements. Daily fasting blood thiamine determinations were made during the 1949 study for an additional subject, B.W.C., who was under a doctor's supervision for a nephritic condition. She consumed a modified diet, containing approximately 2,000 Cal. per day and a daily intake of 2,970 μg of thiamine in period 1, and 2,570 μg of thiamine in period 2. Values were obtained for an additional subject, H.H.Y., during the 1950 study. She consumed a diet containing 2,600 Cal. per day and a daily intake of 6,000 μg of thiamine in period 1, and 5,600 μg of thiamine in period 2. The values obtained for these subjects are shown in figure 1 and table 1. Their blood thiamine concentrations were higher than those of the other subjects throughout the studies.

Values for two ambulatory patients receiving thiamine therapy. J.P., a woman, was taking an intramuscular injection of a multiple vitamin preparation (containing 10 mg of thiamine hydrochloride) once a week. A blood sample, collected one hour after the injection, gave a thiamine value of 13.4 μg per 100 ml of whole blood. Another sample collected the following week, just before the next injection, gave a value of 6.1. H.H., a man, was taking 50 mg of thiamine by mouth every morning. A blood sample was collected some time before lunch. The whole blood thiamine value was 9.5 μg per 100 ml.

Values obtained from laboratory workers on freely selected diets. At intervals during a period of two months blood samples were secured from 5 laboratory workers who were presumably normal and well-fed. None of them took vitamin

preparations. The results, expressed as micrograms of thiamine per 100 ml of whole blood, were: B.W.C., 12 determinations, average: 4.6, range: 3.8–5.2; R.B.D., 10 determinations, average: 4.5, range: 3.7–5.6; C.A.S., 4 determinations, aver-

TABLE 2

Response to a 5-mg oral test dose of thiamine hydrochloride by subjects maintained on a daily thiamine intake of 300 μ g per 1,000 Cal. for 15 days

SUBJECT	BLOOD THIAMINE		URINARY THIAMINE
	Time after test dose		On day of test
	0	1 hr.	
	μ g/100 ml	μ g/100 ml	μ g/24 hr.
M.L.W.	4.2	5.1	195
H.A.L.	4.0	3.8	131
C.A.S.	3.5	3.9	142
R.B.D.	4.6	5.6	376

TABLE 3

Response to a 5-mg oral test dose of thiamine hydrochloride by subjects on a freely chosen diet for one month

SUBJECT	BLOOD THIAMINE					URINARY THIAMINE
	Time after test dose					On day of test
	0	$\frac{1}{2}$ hr.	1 hr.	2 hr.	3 hr.	
		μ g/100 ml				μ g/24 hr.
M.L.W.	4.7	4.7	5.1	4.4	4.2	366 ¹
H.A.L.	4.0	4.8	4.9	4.7	4.6	331
C.A.S.	4.7	5.0	5.0	4.5	4.8	466
R.B.D.	4.6	5.4	5.6	4.9	4.6	318

¹ One voiding lost, 9 hours after test dose.

age: 4.4, range: 3.9–4.9; M.T., 3.6 and 3.5; and E.S., 5.0 and 5.0.

Response to a test dose. On the last day of the 1951 study, the 4 subjects were given a 5-mg test dose of thiamine hydrochloride by mouth after a fasting blood sample had been taken. One hour later a second blood sample was collected. According to the urinary data (Louhi et al., '52) all of the subjects were unsaturated. The results are given in table 2.

A month after the close of the 1951 study, another 5-mg test dose of thiamine hydrochloride was administered to the subjects in the fasting state. After taking the test dose solution, each subject had 6 soda crackers and black coffee. Samples of blood were collected just before the administration of the test dose and one-half, one, two and three hours after the test dose. Results are shown in table 3. All of the subjects showed their maximal value after one hour, and in one case the maximum was reached after one-half hour. There was only one case in which there was a significant increase in the response to the test dose.

DISCUSSION

Values for thiamine in whole blood reported in this paper are somewhat lower than values for adults reported by workers using other thiochrome methods. The average of determinations made during all the "periods 1" was $3.75 \mu\text{g}$ per 100 ml. No subject was in a state of inadequate thiamine nutrition according to the urinary data (Louhi et al., '52). In contrast, Burch et al. ('50) found that in Bataan an average value of $3.7 \pm 0.12 \mu\text{g}$ of thiamine per 100 ml of blood was associated with doubtful cases of beriberi. These workers further found ('50, '52b) that the level of thiamine in the blood of non-symptomatic persons studied in Bataan was $4.0 \pm 0.18 \mu\text{g}$ in 1948 and 4.3 ± 0.09 in 1950, and that the level of thiamine in the blood of well-fed New Yorkers averaged $6.0 \mu\text{g}$ per 100 ml. Friedemann and Kmiecik ('43) reported a mean non-fasting blood thiamine value for 7 women of $5.60 (3.0-9.2) \mu\text{g}$ per 100 ml. These were laboratory workers and assumed to be in good nutrition with respect to thiamine. Greenberg and Rinehart ('45) reported a range of blood thiamine values from 5.0 to $10 \mu\text{g}$ per 100 ml. Their subjects were "normal" laboratory workers and medical students.

However, Chieffi and Kirk ('50) considered whole blood thiamine values ranging from 0 to $2.0 \mu\text{g}$ per 100 ml to be low

and values ranging from 3.5 to 6.0 to be normal. Our average values are all above their "low" classification.

The correlation coefficient between values in whole blood and values calculated for packed cells was very high ($r=0.95$ for 112 pairs of observations). This is to be expected as long as the hematocrit values are within normal limits, as was the case in this experiment. However, the admonition of Hennessy ('47) that hematocrit values should be obtained in case there is a large deviation from normal in cell concentration is worthy of consideration, since, as Goodhart and Sinclair ('39) pointed out, most of the vitamin is contained in the cells.

SUMMARY

Eight women served as subjects in studies designed to follow the daily fluctuations of thiamine in whole blood when the thiamine intake was controlled at 500 and 300 μg of thiamine per 1,000 Cal. for periods of two weeks or more. The diet was constant within each study, the only variable being the level of thiamine from one period to the other.

With an intake of about 500 μg of thiamine per 1,000 Cal. the average fasting blood thiamine ranged from 3.3 to 5.4 μg per 100 ml of whole blood or 7.0 to 11.2 μg per 100 ml of packed cells. When the thiamine intake was lowered to 300 μg of thiamine per 1,000 Cal. the average fasting blood thiamine ranged from 2.8 to 4.6 μg per 100 ml of whole blood or 6.3 to 10.0 μg per 100 ml of packed cells.

The effect of a 5-mg oral test dose of thiamine hydrochloride on the level of thiamine in the blood is presented.

A few values for blood thiamine obtained for people other than experimental subjects are included.

ACKNOWLEDGMENTS

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THE PANTOTHENIC ACID CONTENT OF THE BLOOD AND MILK OF SWINE FED SUPPLEMENTAL LEVELS OF THE VITAMIN¹

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Since the discovery of the importance of pantothenic acid in swine nutrition by Hughes ('42), increasing interest has been shown in levels of this vitamin in various feedstuffs as well as in body tissues and fluids. A limited number of studies of the pantothenic acid content of the blood and milk of swine have been reported in recent years. Luecke et al. ('50) found that blood levels of pantothenic acid in swine seemed to be related to the level of this vitamin which was ingested. Davis et al. ('51) reported the results of pantothenic acid assays of sows' milk which indicated that levels of the vitamin increased as lactation progressed.

The results to be reported in this paper were obtained during the course of a series of experiments carried out at the University of Alberta to investigate the pantothenic acid requirements of Yorkshire swine fed small grain rations over a period of two generations.

EXPERIMENTAL

The experimental animals included 4 groups of gilts and representative suckling pigs from the litters of gilts in each

¹The data reported in this paper are a portion of a thesis presented by Bruce D. Owen as partial fulfillment of the requirements for the degree of Master of Science. Acknowledgments are made to Dr. D. R. Clandinin and Dr. A. R. Robblee and Miss R. Renner for invaluable assistance with assay procedure.

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of these groups. The gilts in lot 1 were fed from the time they were weaned at 8 weeks of age on a basal ration of barley supplemented with protein (meat scraps, soybean oil meal, alfalfa meal), minerals (iodized salt and ground limestone) and vitamins A and D. Levels of three, 6 and 12 mg of synthetic calcium pantothenate per pound of feed were added to the rations in lots 2, 3 and 4, respectively.

The control ration was formulated to be as low in pantothenic acid as was feasible using small grains as a basis, and allowing adequate amounts of protein, minerals and vitamins other than pantothenic acid. The ration fed to lot 1 between the middle of gestation and the end of the lactation period contained 14.6% protein on the basis of Kjeldahl nitrogen determination, and 5.5 μ g of pantothenic acid per gram of feed on the basis of microbiological assay following sample preparation by a method similar to that of Ives and Strong ('46). This is equivalent to 2.5 mg of pantothenic acid per pound of feed. For growing pigs, the National Research Council ('50) recommends 4.7 mg of pantothenic acid per pound of feed, but at no time were any indications of a pantothenic acid deficiency observed in the control pigs.

Blood and milk samples were obtained from each sow at one week and 6 weeks postpartum. At one week and 6 weeks after birth, blood samples were also taken from two pigs, one of each sex, which were selected as representative of each litter. The method of obtaining blood samples from the anterior vena cava was similar to that used by Carle and Dewhirst ('42), while the milk samples were obtained by the use of oxytocin in a method similar to the one employed by Braude et al. ('46). Samples were frozen rapidly at -6°F . and stored until such time as the determinations could be made.

The pantothenic acid content of the blood and milk samples was determined microbiologically using *Lactobacillus arabinosus* 17-5 as the test organism. Assays were conducted on a semimicro scale in which the final volume was 2 ml. The composition of the basal medium used and the assay technique followed were similar to those described by Riesen et al.

('47). The medium differed from that used by these workers in that proline was included.

So far as is known, this type of medium, wherein the casein or peptone is replaced by a solution of pure amino acids, has not been used previously for the assay of pantothenic acid. The medium was found very satisfactory as it yielded extremely low blanks and gave a smooth, repeatable standard curve.

Two procedures were used in the preparation of the blood and milk samples for assay: i.e., autolysis in water for 15 minutes in an autoclave at 121°C., and enzymatic hydrolysis. The latter procedure involved digestion of the sample with a fungal enzyme, mylase P. The preparation of the samples for assay was based on the method used by Ray et al. ('47) with several modifications being made to adapt the method to the purpose of this assay. All samples were assayed in duplicate, and the values reported were obtained in at least two replicate assays. Recoveries in almost all of the cases were between 90 and 110%. As no standardized procedure using chicken liver enzyme and intestinal phosphatase for the release of bound pantothenic acid was available while this work was in progress (György, '50), it was not felt advisable to use this method.

RESULTS

The results of pantothenic acid assays carried out on the various blood samples are shown in table 1.

Enzymatic digestion of the samples gave consistently higher values for pantothenic acid than were obtained by water hydrolysis. The increase in values obtained was considerably greater in the case of sows' blood than in the blood of the young pigs. It may be noted also that the pantothenic acid content of the sows' blood increased as the stage of lactation advanced, while levels of the vitamin decreased in the blood of the young pigs as they grew older. There was no indication that the sex of the young pigs had any influence on the pantothenic acid content of their blood. Although blood levels

tended to correlate with the levels of pantothenic acid in the ration, statistically significant differences between lots were not present insofar as the content of pantothenic acid in the blood was concerned.

TABLE 1
Pantothenic acid content of swine blood

LOT NO. Treatment Time after parturition	1		2		3		4	
	CONTROL		CONTROL + SUPPLEMENTAL CALCIUM PANTOTHENATE 3 mg/lb.		6 mg/lb.		12 mg/lb.	
	1 wk.	6 wks.	1 wk.	6 wks.	1 wk.	6 wks.	1 wk.	6 wks.
	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
Water hydrolysis								
Sow 1	0.18	0.27	0.18	0.20	0.23	0.32	0.24	0.53
Av. of 2 pigs	2.63	0.71	3.14	0.68	3.27	0.76	3.52	0.84
Sow 2	0.15	0.27	0.27	0.27	0.40	0.47	0.41	0.35
Av. of 2 pigs	1.89	1.27	1.58	1.03	5.75	1.40	2.40	1.65
Sow 3					0.24	0.29	0.39	0.55
Av. of 2 pigs					1.72	1.46	5.19	1.02
Av. of all sows	0.16	0.27	0.23	0.24	0.29	0.36	0.35	0.47
Av. of all pigs	2.26	0.99	2.36	0.85	3.58	1.20	3.70	1.17
Enzymatic hydrolysis								
Sow 1	0.55	0.69	...	0.63	0.57	0.73	0.67	0.98
Av. of 2 pigs	3.61	1.09	3.82	1.05	4.27	1.17	4.48	1.22
Sow 2	0.38	0.81	0.61	0.76	0.58	0.94	0.61	0.92
Av. of 2 pigs	2.51	2.08	2.06	1.69	6.66	1.92	2.87	2.25
Sow 3					0.47	0.66	0.59	1.08
Av. of 2 pigs					2.34	1.93	6.01	1.43
Av. of all sows	0.46	0.75	0.61	0.70	0.54	0.78	0.62	0.99
Av. of all pigs	3.06	1.58	2.94	1.37	4.42	1.68	4.45	1.63

Results of assays carried out on the milk samples are shown in table 2.

Analysis of variance according to the method of Johnson ('50) was carried out on the data obtained following water hydrolysis of the samples. The results indicated that the pantothenic acid content of the sows' ration had a very definite effect on the amounts of the vitamin present in the milk, as the milk levels increased consistently and quite markedly with the amount of calcium pantothenate added to the basal ration. As was the case in sows' blood, the panto-

thenic acid content of the sows' milk increased as the lactation period progressed. A possible explanation for the lower values for pantothenic acid obtained on the milk samples following enzymatic hydrolysis will be discussed.

TABLE 2
Pantothenic acid content of sows' milk

LOT NO.	TREATMENT	SOW NO.	TIME AFTER PARTURITION			
			1 week		6 weeks	
			Water	Enzyme	Water	Enzyme
			hydrolysis		hydrolysis	
			$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
1	Control ration (2.5 mg pantothenic acid per pound feed)	1	3.99	3.84	3.63	3.97
		2	7.01	6.34	8.75	6.95
		Av. for lot	5.50	5.09	6.19	5.46
2	Control + 3 mg calcium pantothenate per pound feed	1	6.24	5.71	6.66	6.99
		2	5.92	6.03	7.45	6.15
		Av. for lot	6.08	5.87	7.06	6.57
3	Control + 6 mg calcium pantothenate per pound feed	1	7.81	7.17	9.30	8.87
		2	9.36	10.19	13.23	11.48
		3	7.18	8.13	9.90	7.70
		Av. for lot	8.12	8.50	10.81 ¹	9.35
4	Control + 12 mg calcium pantothenate per pound feed	1	10.14	9.17	17.93	17.15
		2	8.19	9.30	15.78	14.15
		3	10.54	10.78	14.98	12.98
		Av. for lot	9.62 ¹	9.75	16.23 ²	14.76

¹ Significant difference between this and corresponding value in the control lot.

² Highly significant difference between this and corresponding value in the control lot.

DISCUSSION

As is indicated in table 1, the blood levels of pantothenic acid tended to increase in both sows and young pigs with the level of the vitamin in the sows' ration. This trend appeared to be quite regular, although several values were obtained for individual animals which were considerably out of line, and none of the differences was statistically significant. Luecke et al. ('50) observed a similar trend in blood levels of pantothenic acid in swine. The values obtained

following enzymatic digestion of samples were consistently higher than those obtained following water hydrolysis. This would indicate that some of the blood pantothenic acid existed in a bound form, which observation agrees with the work reported by Wright ('43). The increase in values obtained following enzymatic digestion was considerably greater for sows than for young pigs, which possibly indicates that a greater proportion of bound pantothenic acid existed in the blood of the older animals. It may also be noted that the pantothenic acid level in the blood of the sows increased as lactation progressed, while that in the pigs' blood decreased. Although there are no comparable results of other workers with which to compare these findings, a similar occurrence takes place in the case of certain other blood constituents of swine. The work of Grummer et al. ('48) shows a similar trend in blood levels of other members of the B-complex group of vitamins.

The results summarized in table 2 of assays which were conducted on milk samples indicate that the level of pantothenic acid in the sows' ration was reflected in the milk levels of the vitamin. The pantothenic acid in the milk of sows in lot 4 at one week and 6 weeks and in lot 3 at 6 weeks after parturition, was higher by either a significant or highly significant degree than the respective levels in the control lot. Throughout the other lots there was a definite trend toward increased levels of the vitamin in the milk as the level in the ration increased. Differences in milk levels of pantothenic acid tended to be wider than corresponding differences in the blood. This is not surprising, since the blood tends to remain reasonably constant in the level of most nutrients, while secretions and places of storage of various constituents may vary widely in the content of that same nutrient. It was also noted that an increase in milk pantothenic acid levels occurred between one week and 6 weeks after farrowing; an increase which was more marked as the level of the vitamin in the ration was increased. Assays of sows' milk by Davis et al. ('51) also indicated that the level of pantothenic acid

in the milk increased as lactation progressed. Pantothenic acid levels in milk as reported by these workers appear to be lower than those obtained in the present study. This may be explainable on the basis of the different type of basal ration used or by a breed difference in the animals used. However, the differences are probably not of sufficient magnitude to be other than normal variation.

The fact that the values obtained following enzymatic digestion of milk samples were quite consistently lower than those obtained following water hydrolysis is a feature of the milk assay results which is difficult to explain. The differences were greater than those encountered between duplicates of either kind of hydrolysis, so that normal variation is not the explanation. It should be noted here that other workers (Lawrence et al., '46; Davis et al., '51) have reported that little or none of the pantothenic acid in milk is in a bound form, so that the water hydrolysis values obtained in these assays may be considered as maximum. This would indicate that there was perhaps some slight destruction of the vitamin during enzymatic digestion.

SUMMARY

The pantothenic acid content of the blood and milk of sows fed varying levels of pantothenic acid, and of the blood of their suckling pigs, was determined by microbiological assay using *L. arabinosus* as the test organism. In the basal medium, a solution of pure amino acids was used to replace casein or peptone as previously used in pantothenic acid assays. The results illustrated that the pantothenic acid contents of the blood and milk of the sows and of the blood of the suckling pigs were related to the content of the vitamin in the sows' ration. This was particularly true in the case of the milk, where it was found that supplemental levels of 6 or 12 mg of calcium pantothenate per pound of feed fed to the sows resulted in statistically significant increases in the milk levels of the vitamin. Enzymatic digestion using mylase P gave higher pantothenic acid values than water hydrolysis for

sows' and young suckling pigs' blood, but not for the sows' milk.

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FACTORS AFFECTING THE DEVELOPMENT OF ACRODYNIA IN PYRIDOXINE- DEFICIENT RATS

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The first report of a "pellagra-like" condition in rats was made in 1926 (Goldberger and Lillie, '26). Although not recognized as such, this condition was the result of a vitamin B₆ deficiency in rats maintained on a diet which consisted mainly of alcohol-extracted cornmeal. The deficiency syndrome appeared in the animals as evidenced by bilateral symmetrical scaly dermatitis of the ears, extremities, and face. In 1934 and 1935, it was demonstrated that a deficiency of vitamin B₆ produced the symptoms of dermatitis noted by Goldberger and Lillie (György, '34, '35). György suggested that this dietary essential should be named the "rat acrodynia factor" since the lesions of the extremities resembled those observed in human acrodynia, rather than pellagra.

A more detailed description of the rat acrodynia associated with vitamin B₆ deficiency has been given in the literature (Sullivan and Nicholls, '40). Grossly, the initial change is erythema of the dorsa of the paws, most commonly the hind ones, spreading to the plantae, and followed by hyperkeratosis and scaling. The digits next become swollen. Coincident with these changes in the extremities, the same process takes place in the ears, nose, chin, submental region, and occasionally the upper thorax. Although the coat is ill-kempt, very little alopecia is observed until late in the course of

deficiency. Whether involvement of epithelial tissue with subsequent breakdown is a result of secondary infection (Sullivan and Nicholls, '40) or whether it is a primary effect of the deficiency (Wolbach and Bessey, '42) is not yet known.

Several factors have been reported to influence the development of the acrodynia. Within limits, the time of appearance of deficiency symptoms in rats deprived of vitamin B₆ decreased with an increasing protein level in the diet (Cerecedo and Foy, '44). Supplementation of a 15% casein, pyridoxine-deficient diet with methionine or cystine (Cerecedo et al., '48) or with homocystine (Cerecedo and deRenzo, '50) hastened the appearance of these symptoms and increased the severity of the vitamin deficiency syndrome. Pagé and Gingras ('47) have reported that addition of glycine to an 18% casein, pyridoxine-deficient diet provoked a rapid appearance of acrodynia in rats.

The acrodynia of vitamin B₆-deficient rats has been related to the essential fatty acids by several investigators with conflicting conclusions. Quackenbush and associates ('39) reported that the symptoms developed in rats maintained on a pyridoxine-free diet could be alleviated by administration of various natural fats and synthetic esters, the activity of the natural fats for this effect being parallel with their degree of unsaturation. Salmon ('38) has noted that both a heated yeast extract and certain oils are necessary for the maintenance of a normal skin condition in rats, and Birch ('38) has indicated that both fat and water-soluble factors are necessary. In a later study, Salmon ('41) demonstrated that the dermatitis in rats provided with casein, sucrose, salts, carotene, calciferol, alpha-tocopherol, thiamine, riboflavin, and choline could be cured by the administration of pyridoxine, linoleic acid, and pantothenic acid. Quackenbush and associates ('42) later showed that the skin condition which developed in rats fed a highly-purified, fat-free, pyridoxine-free diet could be completely cured by administration of linoleic acid. With subcurative doses of linoleic acid, pyridoxine was

effective, more so in the presence of pantothenic acid than in its absence.

The addition of the vitamin analogue, desoxypyridoxine, decreases the time of appearance, and increases the severity of the acrodynia. With the dietary levels employed, the pattern of the deficiency syndrome appears to be unchanged from that observed by simple pyridoxine deprivation (Stoerk, '50).

The experimental data reported here have been obtained during an investigation of metabolic disturbances in vitamin B₆ deficiency. Since several interesting observations were noted regarding the development of the acrodynia in pyridoxine-deficient rats, and since no correlation was observed between metabolic changes and the appearance of acrodynia, it was felt advisable to describe these findings separately from the data on metabolic changes.

EXPERIMENTAL AND RESULTS

In the experiments to be reported, the analogue, desoxypyridoxine, was employed to facilitate the development of the deficiency syndrome. This was carried out by the addition of 100 μ g desoxypyridoxine per rat per day to the purified diets; control animals were further provided with 50 μ g pyridoxine hydrochloride per rat per day in their food.

Two types of observations have been made with regard to acrodynia: the length of time required for the first clear-cut signs, and the severity of the lesions at the end of a particular interval. Both types are affected by considerable subjective error. The severity of the acrodynia has been graded as slight, moderate or severe in the following ways: slight acrodynia, erythema of the dorsa of the paws with some evidence of swelling; moderate acrodynia, marked erythema and swelling of the dorsa and plantae of the paws with erythema about the nose; severe acrodynia, marked erythema, swelling, and scaling about the paws, nose, and chin, accompanied by open lesions about the same areas.

A consistent observation which has been noted in the numerous experiments performed in this laboratory has been the greater ease with which male albino rats develop acrodynia as compared with females. Not only is the rate of appearance of acrodynia greater in male rats, but the acrodynia is much more severe after a prolonged experimental period.

Supplementation of an incomplete protein diet with certain amino acids

Albino rats of the Wistar strain, with an average initial body weight of 136 gm were housed in individual, screen-bottomed cages, and supplied with food and water ad libitum. The basal diet contained the following ingredients in per cent by weight: gelatin 16, sucrose 70, agar 2, vitamin powder 4, choline 0.2, inositol 0.2, cod liver oil concentrate 0.015 (calculated to supply 45 I.U. of vitamin A and 11 I.U. vitamin D per rat per day), DL-tryptophan 0.44, L-glutamic acid 3.2, and salts mixture 4 (Steenbock and Nelson, '23). The vitamin powder was prepared by thoroughly mixing 800 gm of gelatin with the following amounts (in milligrams) of vitamins: thiamine chloride 100, riboflavin 100, calcium pantothenate 400, nicotinic acid 400, *p*-aminobenzoic acid 400, biotin 20, and folic acid 20. The inclusion of this vitamin powder brings the total protein content of the diet to 20%. It should be noted that this basal diet is devoid of fat, and is referred to later as "fat-free."

In certain cases, as will be indicated, the following amino acid supplements were added to the diet in per cent by weight at the expense of sucrose: DL-methionine 0.55, and DL-histidine 0.4. These supplements made the methionine and histidine contents of the gelatin diet comparable with a diet differing only in that vitamin-free casein replaced the gelatin again in amount to give a total protein content of 20%. All of the animals were provided with 100 µg desoxyypyridoxine per rat per day in their food, and control animals on each diet were further provided with 50 µg pyridoxine per

rat per day in their food. In every case, groups contained 4 male and 4 female rats, and were maintained on experimental diets for a period of 24 days. The results of this experiment are given in table 1.

It would appear that under experimental conditions employing an incomplete protein in the diet, the external sign of pyridoxine deficiency, acrodynia, is not manifested. Even those animals provided with pyridoxine on gelatin-containing

TABLE 1
Effects of an incomplete protein diet on the appearance of acrodynia

DIET	PYRIDOXINE	AVERAGE CHANGE IN BODY WEIGHT	AVERAGE DAILY FOOD INTAKE	ACRODYNIA
		<i>gm</i>	<i>gm</i>	
20% casein	+	+ 55	19.9	none
20% casein	—	— 6	12.9	severe
20% gelatin	+	— 23	18.3	none
20% gelatin	—	— 37	14.1	none
+ methionine	+	— 11	17.9	none
+ methionine	—	— 19	11.0	none
+ histidine	+	— 26	12.5	none
+ histidine	—	— 37	10.4	none
+ methionine	+	— 12	13.6	none
+ histidine				
+ methionine	—	— 29	11.1	none
+ histidine				

diets lost weight. This suggests the possibility that acrodynia develops only under dietary conditions which permit an increase in body weight when vitamin B₆ is furnished. It must be noted that when gelatin, methionine, histidine, glutamic acid, and tryptophan are fed conjointly, the amino acid composition of the diet is still inferior to that of the casein diet and the possibility exists that one or more amino acids, either absent or in low concentration, may be responsible for the absence of the acrodynia.

The effect on acrodynia of supplementing a casein diet with glutamic acid

The basal diet employed in this experiment was that described in the gelatin experiment with the one exception that the gelatin was replaced by vitamin-free casein. A second diet was employed differing in that the total casein content was raised from 20% to 75% by weight at the expense of sucrose. The third diet used was identical with the 20% casein diet but L-glutamic acid was added at a level of 12.1% by weight at the expense of sucrose, thus making it comparable

TABLE 2

The effect on the appearance of acrodynia of dietary L-glutamic acid

DIET	PYRIDOXINE	AVERAGE CHANGE IN BODY WEIGHT	AVERAGE DAILY FOOD INTAKE	ACRODYNIA
		<i>gm</i>	<i>gm</i>	
20% casein	+	+ 28	17.3	none
20% casein	—	— 7	14.4	slight
75% casein	+	+ 4	12.4	none
75% casein	—	— 24	8.4	moderate
20% casein				
+ glutamic acid	+	+ 22	15.0	none
20% casein				
+ glutamic acid	—	— 24	13.1	severe

in glutamic acid content with the 75% casein diet. Again, 100 μ g desoxypyridoxine per rat per day were added to the diet of all animals, and the control animals were further provided with 50 μ g pyridoxine hydrochloride per rat per day in their food. Food and water were supplied ad libitum and animals were maintained on these regimes for 20 days. Each group was comprised of 6 male and 6 female rat with an average body weight of 140 gm. The accrued data on acrodynia are given in table 2.

In the literature, pyridoxine has been related to glutamic acid metabolism, particularly in transamination and decar-

boxylation reactions. From the results of this experiment it would appear that supplementation of a casein-containing diet with L-glutamic acid precipitated the development of acrodynia. This effect was more pronounced when glutamic acid was added alone and in the free form, than when it was added in combination with other amino acids to comprise the 75% casein diet. It has already been indicated that the addition of methionine, cystine, homocystine, or glycine, or increasing the protein level of the diet, will provoke a rapid appearance of acrodynia in rats deprived of pyridoxine. It would seem that another amino acid, glutamic acid, has the same marked effect when supplied with a complete protein.

*The effect on acrodynia of the inclusion of
corn oil in the diet*

Since, in the literature, pyridoxine has been related to fat metabolism, it was felt that adding corn oil to a fat-free, pyridoxine-free diet might have a significant effect on the development of acrodynia. As described in the introduction to this paper, the essential fatty acid, linoleic acid, has been reported to alleviate this external deficiency sign; however, little is known of the effect of including several fatty acids in combination (e.g., corn oil) in a pyridoxine-free diet.

In this experiment, three diets were employed: the 20% casein diet already described, the same diet with corn oil added to a level of 5% by weight at the expense of sucrose, and the 20% casein diet with corn oil added to a level of 20% by weight at the expense of sucrose. In all cases the animals were provided with 100 μ g desoxypyridoxine per rat per day in their food, and control animals on each diet were further provided with 50 μ g pyridoxine hydrochloride per rat per day in their food. Each group was comprised of 5 male and 5 female rats with an average body weight of 211 gm. Each control group was restricted to the same food intake as its comparable deficient group, and all animals were maintained on these dietary regimens for a period of 28 days. The results of this experiment are recorded in table 3.

It was found that corn oil had no effect on the rate of appearance of the acrodynia (21 days), but had a marked effect in preventing the development of a severe degree of the deficiency sign. The effect of corn oil in apparently preventing severe acrodynia is consonant with the concept of an interrelationship between vitamin B₆ and the essential fatty acid, linoleic acid.

TABLE 3

The effect on acrodynia of adding corn oil to a fat-free diet

DIET	PYRIDOXINE	AVERAGE CHANGE IN BODY WEIGHT	AVERAGE DAILY FOOD INTAKE	ACRODYNIA
		gm	gm	
fat-free	+	— 9	11.2	none
fat-free	—	— 25	11.2	severe
5% corn oil	+	+ 12	10.9	none
5% corn oil	—	— 35	10.9	slight
20% corn oil	+	+ 32	11.3	none
20% corn oil	—	— 17	11.3	slight

Hormonal effects on the development of acrodynia

The results of several experiments studying the interrelation of pyridoxine and certain hormones will be briefly summarized. Work in this laboratory has suggested that acrodynia does not develop in adrenalectomized rats maintained on the 20% casein, 20% corn oil diet previously described, when provided with desoxypyridoxine but no pyridoxine. In contrast with this observation, similarly treated ovariectomized rats, and intact animals subcutaneously injected with 4 µg of an oestrogenic preparation¹ in olive oil per rat per day, exhibited a typical pyridoxine-deficiency syndrome. It has been further noted that subcutaneous injection of an adrenocorticotrophic hormone preparation,² or of an adrenal cortical extract preparation,³ were without effect on the de-

¹ Ayerst, McKenna, Harrison.

² Armour Laboratories 212-103.

³ Connaught Medical Research Laboratories.

velopment of acrodynia. Daily subcutaneous administration of a growth hormone preparation,⁴ on the other hand, appeared to aggravate the pyridoxine deficiency.

SUMMARY

Several factors have been noted during investigations with pyridoxine which affect the development of the external deficiency sign, acrodynia.

1. Rats maintained on a diet containing gelatin as the source of nitrogen do not exhibit acrodynia when deprived of pyridoxine and provided with desoxypyridoxine, even when this diet is supplemented with tryptophan, glutamic acid, methionine, and histidine.

2. Supplementation of a diet containing casein with glutamic acid appears to provoke the development of acrodynia in pyridoxine-deficient rats.

3. The inclusion of corn oil in a fat-free, pyridoxine-free diet had no effect upon the rate of appearance of acrodynia, but did appear to prevent the development of a severe stage of the external deficiency sign.

4. In all of the groups studied the appearance of acrodynia was more frequent and more severe in male rats than in the females.

5. Adrenalectomy appeared to prevent the appearance of acrodynia, whereas ovariectomy, ACTH administration and adrenal cortical extract administration were without effect. Administration of a growth hormone preparation appeared to aggravate a pyridoxine deficiency.

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⁴ Armour Laboratories 22KR1.

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INTERMEDIATES FORMED DURING THE DIGESTION OF TRIGLYCERIDES

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ONE FIGURE

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It seems probable that the cleavage of triglycerides in the intestinal tract involves a series of step-wise reactions from triglyceride (TG) to diglyceride (DG) to monoglyceride (MG) and possibly to glycerol (G), with fatty acid (FA) being released at each step. Such DGs could be either the 1,3- or 1,2-isomer, while the MGs could be either the 1- or 2-isomer depending on whether the reaction resulting in the removal of fatty acid(s) from the glyceride molecule are random or selective. On the basis of the composition of the lipids recovered from the thoracic duct of rats fed tagged TGs, Reiser et al. ('52) concluded that the major portion of ingested fat was hydrolyzed to MGs prior to absorption. The high hydroxyl value of the lipids isolated from the lumen of the intestinal tract of rats was interpreted by Frazer and Sammons ('45) as showing the presence of MGs. Although no experimental details are given, a later paper states that MGs were found in the intestinal tract of human subjects (Frazer, '47). Longenecker ('46) has referred to unpublished work carried out by him which demonstrated the formation of MGs and DGs in the intestinal tract during the digestion of fat. The recent development of several new methods for studying partial glycerides, some of which are reported here for the first time, has enabled us to demonstrate with certainty the presence of MGs and DGs in the intestinal tract as the

result of lipolytic action. Moreover, evidence has been obtained which indicates a selective hydrolysis resulting in the formation of 1,2-DGs and 2-MGs.

EXPERIMENTAL

Male rats weighing 100 to 200 gm were fasted 48 hours and then were given 1.5 ml of fat by stomach tube. The animals were sacrificed at the time intervals given in the individual experiments. The intestines, and in some instances the stomach, were removed separately and the contents flushed out with ethyl ether. The ether solution was immediately dried with sodium sulfate and filtered. Separate experiments established that lipase action ceased completely at this point. The solvent was removed under vacuum at 40°C.; the lipid residue was weighed and then taken up in chloroform. This solution of lipids was divided into two equal fractions. One fraction was washed with water and dried with sodium sulfate preliminary to analysis. The other fraction was treated with perchloric acid to convert the 2-MGs to 1-MG. The sample was then washed with water, dried with sodium sulfate, and analyzed.

Under the experimental conditions employed, 200 to 300 mg of lipid were usually recovered from the lumen of the intestine of a rat which had been fed TG two to 4 hours earlier. The small quantity of material available for analysis necessitated the modification of several standard macro methods. The procedure used for determining MGs was essentially that described by Pohle and Mehlenbacher ('50) except that the solution concentrations were reduced. With this method pure TGs, 1,2-DGs, 1,3-DGs, and 2-MGs yielded blank values of up to 1.5% apparent 1-MG. Thus, MG values below 1.5% are considered not to be significant under the experimental conditions employed here. Similar observations with respect to such blank values have been reported by Kummerow and Daubert ('50). With increasing amounts of 1-MG in the lipid being studied, the size of the sample used for analysis can be reduced and hence the non-specific reaction of TGs

and DGs with periodic acid becomes of less significance. On known mixtures of glycerides, it was found that when the concentration of 1-MG in the lipid is in excess of 6%, the experimentally obtained values are in close agreement with the true values. Although 2-MG does not react with periodic acid, it was possible, by determining the amount of 1-MG initially present, isomerizing the 2-MG to 1-MG, and then again analyzing for 1-MG, to calculate by difference the amount of 2-MG initially present.

Free FAs were determined by titration. Iodine values were determined by a micro adaptation of the Wijs method. Hydroxyl values were obtained by a modification of the Zerevitinoff active hydrogen method using lithium aluminum hydride as the reagent (Hochstein, '49). The quantity of evolved hydrogen was corrected for that contributed by the free FA; the residual value was then used in calculating the hydroxyl value of the sample. The quantity of DG present was calculated from the hydroxyl value remaining after subtracting from the total hydroxyl value the number of hydroxyl units equivalent to the total MG present.

Craig separation runs were made in a 54-tube apparatus using heptane-80% ethanol as the solvent pair (Zilch and Dutton, '51). Under these conditions the MG fraction peaks at tube 9, free FAs at tube 40, and the DGs and TGs at tube 52. The partition coefficients of 1-MGs and 2-MGs were found to be essentially identical.

Studies with synthetic 2-MG demonstrated that conversion to the 1-isomer could be brought about by a number of different methods. Of these, the one chosen as being the most efficient, and yet causing the minimum of hydrolysis and interesterification reactions, was treatment with perchloric acid. Essentially, the method consists of dissolving the lipids in chloroform so as to obtain approximately a 1% solution of MGs. To this is added sufficient 55% perchloric acid to obtain a ratio of one part perchloric acid for every 4 parts of MG. The solution is mixed and allowed to stand at room temperature for 30 minutes. Under these conditions there is

a 70 to 85% conversion of the 2-isomer to the 1-isomer. Since this treatment does not bring about complete isomerization, the values for total MG, as determined by the periodic acid method, are minimal. The variation in the extent of isomerization makes it impracticable to correct the observed values. On the other hand, the DG values will be maximal, since the hydroxyl value from which the quantity of DG is calculated must be corrected for the quantity of MG present.

Although not used as the method of isomerization, heat also causes a conversion of 2-MG to 1-MG. Thus holding a sample of 2-MG at a temperature of 100°C. for three hours will cause as much as 80% isomerization. At lower temperatures, the rate of conversion is slower. The variations in the extent of isomerization, and the accompanying hydrolytic and esterification reactions, when heat is used, make it an unsuitable method.

RESULTS

In the first series, 12 rats were given 1.5 ml of partially hydrogenated vegetable oil. Groups of three rats each were sacrificed at zero, two, three, and 4 hours. The intestinal and stomach samples of each group of three animals were pooled separately. The MG and free FA content of the isolated lipids are given in table 1.

It will be noted that little or no MG or FA was present in the stomachs of these animals. Similarly, appreciable quantities of digestion products or intermediates could not be demonstrated in the intestines of the zero hour animals. In the intestines of the animals sacrificed at the end of the other time periods considerable amounts of MGs and free FA were present. An appreciable portion of the MGs was of the 2-configuration.

In order to have larger samples for analysis, each of 20 rats was administered 1.5 ml of partially hydrogenated vegetable oil. All of the animals were sacrificed at the end of three hours. The lipids recovered from the lumen of the intestines of these rats were pooled into a single sample.

Analyses of the isomerized and unisomerized samples are given in table 2.

The Craig countercurrent distribution curves are given in figure 1 and the average composition of the lipids as calculated from the curves is presented in table 2. The dotted

TABLE 1

The monoglyceride (MG) and free fatty acid (FA) content of the lipids recovered from the lumen of the stomachs and intestines of rats at various intervals following the feeding of triglyceride¹

HOURS	FREE FA	1-MG	TOTAL MG	2-MG ²
	%	%	%	%
<i>Stomach</i>				
0	2.9	0.7	0.6	0
2	3.0	1.0	0.9	0
3	4.8	1.4	1.5	0
4	1.4	1.8	2.3	0.5
<i>Intestine</i>				
0	4.2	1.8
2	21.5	3.4	7.5	4.1
3	24.5	3.2	8.2	5.0
4	35.7	9.6	16.4	6.8

¹ A 75/25 mixture of soybean oil and cottonseed oil hydrogenated to an iodine value of 80.

² Determined by difference.

TABLE 2

The percentage composition of the lipids recovered from the lumen of the intestines of rats fed triglyceride¹

	CHEMICAL ANALYSES	CRAIG SEPARATOR
1-Monoglyceride	3.2	
Total monoglyceride	7.5	10.6
2-Monoglyceride	4.3 ³	..
Free fatty acid	15.5	13.6
Hydroxyl value ²	57	..
Diglyceride	36	} 75.5
Triglyceride	41 ³	

¹ A 75/25 mixture of soybean oil and cottonseed oil hydrogenated to an iodine value of 80.

² Theoretical hydroxyl values for partial glycerides of this fatty acid composition are 317 for monoglyceride and 91 for diglyceride.

³ Determined by difference.

lines on the curves are the areas used in calculating the quantities of FAs, DGs, and TG

The material contained in tubes zero to 20 from the solvent separation of the unisomerized sample was combined into a single sample, hydrogenated to a zero iodine value, and an x-ray diffraction pattern obtained. The pattern was typical

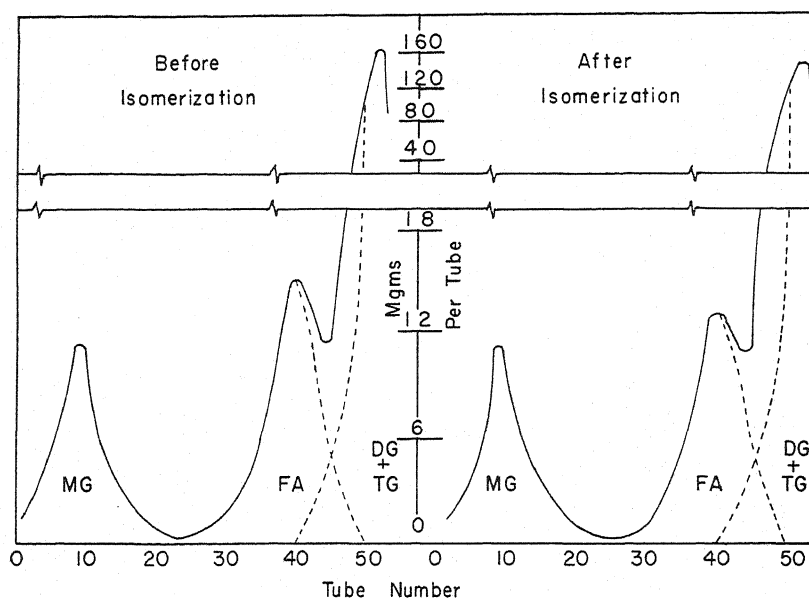


Fig. 1 Countercurrent distribution curves of the lipids isolated from the intestines of rats fed triglyceride.

of that for 1-MG. The presence of 2-MG in the sample will not influence the pattern since during hydrogenation 2-MG is converted to 1-MG.

The presence of MG in the original samples and in the MG fraction isolated by countercurrent distribution was confirmed further by infrared spectrophotometry. The regions of the spectrum where MGs show characteristic absorption are 9.5 and 3 μ .

In the final series, each of 20 rats was given 1.5 ml of 2-oleyldipalmitin (POP), iodine value = 30. All animals were

sacrificed at the end of three hours. The lipids recovered from the lumen of the intestines of these rats were pooled into a single sample. Analyses of the isomerized and unisomerized samples are given in table 3.

The samples were then processed by countercurrent distribution. The distribution curves, although not shown here, were similar to those in figure 1. The MGs contained in tubes zero to 20 were combined into a single sample. The iodine value of this material was 64. The lipids contained in

TABLE 3

The percentage composition of the lipids recovered from the lumen of the intestines of rats fed 2-oleyl-dipalmitin

CHEMICAL ANALYSES	
1-Monoglyceride	6.5
Total monoglyceride	13.3
2-Monoglyceride	6.8 ²
Free fatty acid	19.7
Hydroxyl value ¹	65
Diglyceride	22
Triglyceride	45 ²
Iodine value of monoglycerides	64
Iodine value of fatty acids	5

¹ The theoretical hydroxyl value of 326 for monoolein and 94 for 1-palmito-2-olein used in these calculations.

² Determined by difference.

tubes 35 to 43 (these would be predominantly free FA with some DGs and TGs) were combined into a single sample. The free FAs were removed by washing with a dilute solution of potassium carbonate. The potassium carbonate wash was acidified and the FAs were recovered by extraction with petroleum ether. These FAs had an iodine value of 5.

DISCUSSION

The analytical techniques used in these experiments had certain limitations which could not be completely overcome. As mentioned, the periodic acid reagent indicates a small amount of apparent MG in fats known to be free of MG. For

this reason the values reported in table 1 for the MG content of the lipids of the stomachs are probably spurious values. The only exceptions to this may be the values at the end of 4 hours. In the case of the figures for the intestinal samples in table 1, except for the zero hour samples, the MG values obtained are well in excess of any limitations the method may have. This is particularly true of values in excess of 6% MG, where the values will be accurate as well as real.

The method of converting 2-MG to 1-MG was not quantitative. Under the conditions employed, the treatment of a pure sample of 2-MG with perchloric acid will cause an 85% conversion to the 1-isomer. However, when TG, DG, and FA are also present this conversion may be as low as 70%. Therefore, in the samples studied here it is possible that only a 70% conversion was obtained. That the isomerization is incomplete is confirmed by the higher values for MG obtained by countercurrent fractionation. Moreover, the MG fractions obtained from the Craig runs on the isomerized samples when analyzed for MG by the periodic method show only 70% 1-MG. The uniformity of the MG fraction of the Craig curves and the fact that 1- and 2-MG have the same partition coefficients in the solvent pair used, indicate the remaining portion of the sample to be 2-MG. It is unlikely that there were other materials in the samples that would concentrate in the zero to 20-tube region.

These limitations in the methods made it desirable to have an additional confirmation of the presence of 2-MG. For this reason the experiment using POP was carried out. If the hydrolysis to MG was a random reaction, then the MG fraction should consist of two parts monopalmitin and one part monoolein. Such a mixture would have an iodine value of 24. If only 1-MG were formed then the iodine value of the MG fraction would be zero, while if only 2-MG were formed then the iodine value would be that of monoolein, 71. The iodine value of 64 that was obtained on the isolated MG was only 10% less than that for pure monoolein. Similarly if the hydrolysis of TGs was a random reaction then the free FAs

should consist of two parts palmitic and one part oleic acid. Such a mixture would have an iodine value of 32. The iodine value of 5 found for the free FAs indicates the predominant free FA to be palmitic acid. Thus from the composition of the MGs formed and the FAs released following the feeding of POP, it appears that the MG resulting from the *in vivo* hydrolysis of TG is predominantly the 2-isomer.

The results obtained in the experiment where POP was fed indicate that some 90% of the MGs formed were of the 2-configuration. However, in none of the experiments did the 2-isomer, as measured by chemical means, ever constitute more than 70% of the total MG present and was usually considerably less. Thus it seems that even in the samples that were not treated with perchloric acid, some isomerization had taken place. Although a small part of this isomerization may have taken place during isolation of the samples, isomerization in the intestine also seems to take place. The increasing amount of the 1-isomer found in the intestinal tract with increasing time (see table 1) indicates such a conversion. An interesting contrast is that in *in vitro* digestion experiments with pancreatic lipase, using the periodic acid method of analysis, we have been able to identify only 1-MG.

The net effect of the limitations described results in the obtaining of minimum values for the MG content. The presence of these minimal quantities of MG has been established by the periodic acid method and countercurrent separation and their presence qualitatively confirmed by x-ray diffraction pattern and absorption in the infrared. Future refinements of technique should have the effect of showing more total MG and 2-MG, and less DG.

Since the MGs initially formed seem to be predominantly of the 2-configuration, as much as 90% being indicated by the experiment when POP was fed, it is reasonable to assume that the DGs formed are the 1,2-isomer. Unfortunately, there is not a suitable method for distinguishing 1,2-DGs from 1,3-DGs.

SUMMARY

1. Lipids recovered from the lumen of the intestines of rats following the feeding of triglyceride were analyzed for monoglyceride by periodic acid before and after isomerization with perchloric acid (this causes a conversion of 2-monoglyceride to 1-monoglyceride), for free fatty acid, and for hydroxyl value. The lipids were also fractionated by the Craig separator and the presence of monoglycerides confirmed by x-ray diffraction patterns and infrared absorption.

2. Appreciable quantities of monoglyceride and diglyceride are formed and accumulate in the lumen of the intestinal tract during the digestion of fat. These glycerides constituted as much as 16 and 36%, respectively, of the total lipids. A portion of the monoglyceride found was of the 2-configuration, and the data indicate that almost all of the monoglycerides formed were initially the 2-isomer. It is likely that the diglycerides formed are of the 1,2-configuration.

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STUDIES OF LIPOGENESIS IN CERTAIN B-VITAMIN DEFICIENCIES¹

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There is evidence to suggest that many vitamins of the B-complex are concerned with fat metabolism (McHenry and Cornett, '44). The role of choline as a lipotropic factor is well established (Best and Lucas, '43; McHenry and Patterson, '44). It has also been shown that high fat diets spare dietary thiamine (Evans and Lepkovsky, '35; Banerji, '40), and that large doses of dietary biotin may produce cholesterol-rich fatty livers (Gavin and McHenry, '41). Coenzyme A, which contains pantothenic acid (Lipman et al., '47), has been found to be involved in the synthesis of ergosterol and fatty acids from acetic acid in yeast (Klein, '51). The accumulation of cholesterol in livers of rats fed a cholesterol-rich diet was found to be depressed in pantothenic acid deficiency (Morgan and Guehring, '51). In addition, Cheldelin et al. ('51) have reported that livers from pantothenic acid-deficient rats fail to oxidize butyrate and caproate.

Other workers have demonstrated conclusively that acetate is a precursor of fatty acids and cholesterol (Rittenberg and

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Bloch, '45; Bloch, Borek and Rittenberg, '46). This study deals with the effect of thiamine, biotin, pantothenic acid and choline deficiencies upon the incorporation of acetate carbon into fatty acids and cholesterol of liver, blood, heart, and adrenals. The endeavor suggests that there is no defect in lipogenesis in any of these deficiencies.

MATERIALS AND METHODS

Groups of young male rats were given one of the diets shown in table 1. Crystalline vitamins were added in the following quantities per kilogram of diet: riboflavin, 8 mg;

TABLE 1
Composition of diets

	A	B	C
	%	%	%
Casein (vitamin-free)	18	20	12
Egg white	72.5	70.5	78.8
Dextrose	3	3	3
Corn oil	2	2	2
Cod liver oil	0.3	0.3	0.3
Choline chloride	0.2	0.2	0.2
Cystine	4	4	4
Salts ¹			

¹ Hegsted, Mills, Elvehjem and Hart ('41).

pyridoxine, 4 mg; calcium pantothenate, 20 mg; niacin, 20 mg; and thiamine, 4 mg. Diet A was fed ad libitum to the control rats. For production of thiamine and pantothenic acid deficiency the respective vitamins were omitted from diet A. Biotin deficiency was produced by diet B, and choline deficiency by diet C. The control groups which were pair-fed with the thiamine, pantothenic acid and choline-deficient rats were given the same diet as the deficient rats with addition of the missing vitamins; the rats pair-fed with the biotin-deficient rats were offered diet A.

A summary of the experimental course of the animals used in this study is shown in table 2. After the characteristic signs

of deficiency had developed, each rat was given daily over three days 0.1 millimoles of radioactive carboxyl-labeled acetate mixed in the food per day per 100 gm of body weight. At the end of the three days of feeding, the animals were killed and their liver, heart, adrenals and blood serum examined. The organs were digested at 70°C. during two hours in 5 ml of 30% KOH, and 5 ml alcohol. After saponification,

TABLE 2
Course of deficiency

DIET	NO. OF RATS	WEIGHT AT BEGINNING OF EXPER. <i>gm</i>	TIME ON DIET <i>weeks</i>	WEIGHT AT BEGINNING OF TEST <i>gm</i>	DAILY FOOD INTAKE DURING TEST <i>gm</i>
Normal	20	40	5-6	135	13.7
Pantothenic acid deficiency	20	55	8-12	99	6.7
Pair-fed	16	55	8-12	124	6.9
Thiamine deficiency	11	53	3-4	90	4.8
Pair-fed	12	53	3-4	106	5.0
Biotin deficiency	11	45	6-7	121	10.0
Pair-fed	13	46	6-7	143	10.0
Choline deficiency	11	40	2-3	98	11.0
Pair-fed	12	39	2-3	99	11.1

5 ml of 6N HCl were added. The fatty acids and cholesterol were extracted with ether, the ether evaporated, and the residue transferred in small centrifuge tubes. After the addition of 3 ml of a 1:1 alcohol-acetone mixture, cholesterol was precipitated with digitonide. On the following day the precipitate was washed with acetone-ether (2:1) and then ether, and the fatty acids of the liver determined gravimetrically in the supernatant solution. The precipitate was dried and an aliquot examined for cholesterol according to the methods of

Sperry ('38) and Sperry and Webb ('50). A weighed amount of the dried fatty acids and the part of the cholesterol-digitonide which was not used for cholesterol determination was placed on planchets and counted for radioactivity with an end-window Geiger-Muller counter.

The administration of 1.0 millimoles of acetate per 100 gm of rat per day to one-half the pantothenic acid-deficient and control groups did not significantly affect the radioactivity of the fractions studied, and these data are included in the average for the groups of this study.

RESULTS

Thiamine deficiency

The data obtained with thiamine-deficient rats are presented in table 3. Marked differences were not found in the fatty acids and cholesterol concentrations of the liver, heart, and blood serum between thiamine-deficient rats and their ad libitum and pair-fed controls. A significant decrease in total incorporation of radioactive carbon in liver fatty acids and cholesterol and in heart cholesterol was noted in the deficient animals as well as in the pair-fed controls, whereas the specific activity (counts per minute per milligram fatty acids and cholesterol) remained markedly constant in all groups. The differences in total activity are, therefore, due to the diminished total amount of these substances. It is noteworthy that the pair-fed controls behaved like the deficient animals, which suggests that it is lack of calories rather than thiamine deficiency which caused the depression. The total and relative cholesterol content of the adrenals was considerably diminished in the animals with restricted food intake. The pair-fed group also showed a diminished incorporation of radioactive carbon in adrenal cholesterol, even on a weight basis, which differed significantly from the normal controls.

Pantothenic acid deficiency

Table 4 shows that pantothenic acid deficiency does not affect fatty acid and cholesterol concentrations of the liver,

TABLE 3
Thiamine deficiency
 (Weight, fatty acid and cholesterol content of liver, heart, adrenals, and blood serum)

CONDITION	NO. OF RATS	LIVER			HEART		ADRENALS		BLOOD SERUM	
		Fatty acids, gm		Cholesterol, mg	Cholesterol, mg		Weight		Cholesterol, mg	
		total	per 100 gm		total	per 100 gm	mg	total	per 100 gm	mg %
Normal	10	0.202	2.56	18.12	242	0.64	117	0.50	1579	49
Deficient	11	0.071	2.04	8.02	211	0.43	114	0.44	1410	52
Pair-fed	12	0.092	2.05	9.45	210	0.50	115	0.35	1158	44

Counts per minute/100,000 c/m given, means and standard deviations										
CONDITION	LIVER			HEART		ADRENALS		SERUM		
	Fatty acids		Cholesterol	Cholesterol		Cholesterol		Cholesterol		
	per mg	total		per mg	total	per mg	total	per mg	total	per mg
Normal	12 ± 5.5	2068 ± 965	22 ± 5.0	341 ± 74	11 ± 2.3	7 ± 1.6	15 ± 6.9	5 ± 2.4	15 ± 4.9	15 ± 4.9
Deficient	15 ± 8.8	1095 ± 517	20 ± 5.0	163 ± 46	9 ± 4.4	4 ± 2.3	11 ± 7.2	4 ± 2.9	16 ± 3.9	16 ± 3.9
Pair-fed	14 ± 4.3	1242 ± 488	20 ± 4.2	186 ± 65	9 ± 3.1	5 ± 1.9	10 ± 3.9	3 ± 1.7	17 ± 8.5	17 ± 8.5

heart, and blood serum, although the total incorporation of C^{14} in these lipids is reduced. The differences in the specific activity, however, were small and insignificant. The adrenals of the deficient animals contained considerably less, and those of the pair-fed controls much more cholesterol than those of the normal animals. As in the case of thiamine deficiency, both deficient and pair-fed rats had a low specific activity in adrenal cholesterol, and the total counts were normal in the pair-fed, and low in the deficient groups.

Biotin deficiency

In this type of deficiency great differences in the fatty acid and cholesterol concentration of the liver, heart, and blood serum were not observed between the deficient animals and their ad libitum and pair-fed controls (table 5). In contrast to the thiamine-deficient and the pantothenic acid-deficient rats incorporation of C^{14} into liver fatty acids by deficient and pair-fed rats was normal, and only the cholesterol synthesis was reduced. Again there is no specific effect of deficiency, although the better food intake in the deficient group slightly modified the results. Adrenal cholesterol concentration was not affected by biotin deficiency, although the incorporation of radiocarbon in adrenal cholesterol was found to be diminished in both the deficient and pair-fed rats.

Choline deficiency

As was expected a considerable increase in the liver fatty acids was found in the choline-deficient animals (table 6). The choline content of the diet of the pair-fed group (which differed from that of the normal control group by its low protein content) did not entirely prevent the rise in fatty-acid content above normal levels. Both deficient and pair-fed animals showed a diminished cholesterol content and concentration in the heart, adrenals, and blood serum, liver cholesterol being subnormal in the pair-fed group.

TABLE 4
Pantothenic acid deficiency
 (Weight, fatty acid and cholesterol content of liver, heart, adrenals, and blood serum)

CONDITION	NO. OF RATS	LIVER		HEART		ADRENALS		BLOOD SERUM	
		Fatty acids, gm		Cholesterol, mg		Cholesterol, mg		Cholesterol	
		total	per 100 gm	total	per 100 gm	total	per 100 gm	total	mg %
Normal	24	0.194	2.46	18.25	231	0.68	123	0.47	1478
Deficient	20	0.101	2.25	9.94	221	0.57	125	0.36	1037
Pair-fed	16	0.107	2.32	9.52	207	0.59	113	0.66	1990

CONDITION	LIVER			HEART		ADRENALS		SERUM	
	Fatty acids			Cholesterol		Cholesterol		Cholesterol	
	per mg	total	per mg	per mg	total	per mg	total	per mg	total
Normal	13 ± 4.6	2238 ± 875	21 ± 6.1	13 ± 4.7	8 ± 3.2	16 ± 7.1	7 ± 3.5	19 ± 7.4	19 ± 7.4
Deficient	11 ± 0.9	1034 ± 376	20 ± 5.0	12 ± 5.1	6 ± 3.0	12 ± 5.8	4 ± 3.1	22 ± 9.2	22 ± 9.2
Pair-fed	11 ± 0.9	1274 ± 351	18 ± 5.6	11 ± 7.5	7 ± 3.7	9 ± 4.7	7 ± 3.5	19 ± 14.4	19 ± 14.4

Counts per minute/100,000 c/m given, means and standard deviations

TABLE 5
Biotin deficiency
 (Weight, fatty acid and cholesterol content of liver, heart, adrenals, and blood serum)

CONDITION	NO. OF RATS	LIVER			HEART		ADRENALS		BLOOD SERUM	
		Fatty acids, gm		Cholesterol, mg	Cholesterol, mg	Weight, mg	Cholesterol, mg		Cholesterol	
		total	per 100 gm	total	per 100 gm		total	per 100 gm		mg %
Normal	10	0.202	2.56	18.12	242	0.64	117	0.50	1579	49
Deficient	11	0.142	2.58	13.04	237	0.66	115	0.65	1547	42
Pair-fed	13	0.146	2.24	13.52	208	0.66	105	0.59	1730	45

CONDITION	LIVER			HEART		ADRENALS		SERUM	
	Fatty acids		Cholesterol	Cholesterol		Cholesterol		Cholesterol	
	per mg	total	per mg	total		per mg	total	per mg	
Normal	12 ± 5.5	2068 ± 965	22 ± 5.0	341 ± 74	11 ± 2.3	7 ± 1.6	15 ± 6.9	5 ± 2.4	15 ± 4.9
Deficient	12 ± 4.5	1698 ± 701	19 ± 6.4	251 ± 93	9 ± 3.9	6 ± 2.7	11 ± 6.0	8 ± 5.2	17 ± 5.6
Pair-fed	10 ± 4.9	1405 ± 599	17 ± 9.9	224 ± 47	8 ± 3.3	5 ± 2.1	9 ± 3.4	6 ± 4.2	12 ± 6.7

Counts per minute/100,000 c/m given, means and standard deviations

TABLE 6

Choline deficiency
(Weight, fatty acid and cholesterol content of liver, heart, adrenals, and blood serum)

CONDITION	NO. OF RATS	LIVER			HEART		ADRENALS		BLOOD SERUM	
		Fatty acids, gm		Cholesterol, mg	Cholesterol, mg		Weight		Cholesterol	
		total	per 100 gm	total	total	per 100 gm	total	per 100 gm	total	mg %
Normal	10	0.202	2.56	18.12	0.64	117	0.50	1579	49	
Deficient	11	0.715	11.92	14.95	0.46	103	0.26	1149	30	
Pair-fed	12	0.190	3.22	10.20	0.44	87	0.28	1313	31	

CONDITION	LIVER			HEART		ADRENALS		SERUM	
	Fatty acids		Cholesterol	Cholesterol		Cholesterol		Cholesterol	
	per mg	total	per mg	per mg	total	per mg	total	per mg	total
Normal	12 ± 5.5	2068 ± 965	22 ± 5.0	11 ± 3.3	7 ± 1.6	15 ± 6.9	5 ± 2.4	15 ± 4.9	
Deficient	8 ± 4.6	6063 ± 3040	22 ± 4.8	11 ± 3.3	5 ± 4.4	11 ± 4.4	3 ± 1.8	20 ± 8.0	
Pair-fed	9 ± 2.2	1741 ± 766	22 ± 3.6	9 ± 2.4	4 ± 0.7	9 ± 3.0	3 ± 1.6	18 ± 12.1	

Counts per minute/100,000 c/m given, means and standard deviations

It is noteworthy that no significant difference was found in the specific activity of liver fatty acids and the liver, heart, and serum cholesterol in deficient and normal rats. The total amount of radioactivity was significantly higher in the liver fatty acids of the deficient rats and significantly lower in liver cholesterol of the pair-fed group as a consequence of the higher fatty acid content in choline deficiency and the lower cholesterol content in the pair-fed rats. The total activity of heart and adrenal cholesterol was diminished in the deficient and pair-fed groups. As in the case of other deficiencies, the total C^{14} content of the adrenal cholesterol was low in the deficient and pair-fed groups.

DISCUSSION

The incorporation of radiocarbon from acetate into fatty acids and cholesterol of the liver, heart, and serum of rats was not *specifically* altered by thiamine, pantothenic acid, biotin, or choline deficiencies. There were, however, significant variations in the *total* incorporation of radiocarbon in these organs in some of these deficiencies. There was an increase in total radioactivity in the fatty acids of the liver in choline deficiency, a decrease in that of the liver fatty acids and cholesterol in thiamine and pantothenic acid deficiencies, and a decrease in that of liver cholesterol in biotin deficiency. In all cases except choline deficiency, the pair-fed controls presented similar findings, suggesting that this effect was due to the diminished food intake. It is of interest to note that the greatest decrease in incorporation occurs in thiamine deficiency which is accompanied by the most severe voluntary restriction of food intake (table 2). In this deficiency, the incorporation of C^{14} , even in heart cholesterol, is significantly depressed. In pantothenic acid deficiency the food intake is less drastically reduced, and the decrease in radioactivity of total heart cholesterol is on the borderline of statistical significance. On the other hand, in biotin deficiency, with an almost normal food intake, no significant decrease in total radioactivity was found in liver fatty acids

with reduction only of total liver cholesterol activity. This finding is not in agreement with the claims of Curran ('50) based on 4 rats only, that the synthesis of cholesterol is normal and that of fatty acids slightly increased in this deficiency.

The findings with choline deficiency are of interest in view of the fact that the rate of accumulation of radioactivity exceeded the rate of accumulation of total fat. Two to three weeks are required to produce the observed increase in liver fat while only three days of feeding radioacetate labeled the increased depot of fat as a normal specific activity. This suggests that the inability to metabolize newly synthesized fat becomes increasingly difficult with progressive choline deficiency.

The lack of a specific effect of pantothenic acid deficiency is puzzling, since a specific decrease in incorporation of radio-carbon from acetate might be expected on the basis of the proven role of coenzyme A in fatty acid synthesis in bacteria (Stadtman, '50). The findings of Olson and Kaplan ('48) that even prolonged pantothenic acid deficiency does not completely deplete the rat organism of its coenzyme A content lends an explanation to our results. One might postulate that a certain minimum level of coenzyme A is essential for certain metabolic processes such as lipogenesis, and if this minimum (about one-third normal) is maintained, no metabolic defect is demonstrable. One might postulate further that lowering of coenzyme A below this level as a result of prolonged deficiency results in death rather than further derangement of lipogenesis or other coenzyme A-dependent reactions. These postulates are not necessarily inconsistent with the findings that deficient rats are unable, even at an earlier stage of pantothenic acid deficiency, to utilize a coenzyme A-dependent reaction if a load is imposed, i.e., the acetylation of PABA (Riggs and Hegsted, '48).

Our findings with adrenal cholesterol deserve a further comment. It is believed that adrenal cholesterol may serve as a precursor of cortical hormones (Long, '47) and that its



amount reflects the reaction of the organism to "stressor agents." During the period of initial alarm, the concentration of cholesterol in the adrenals decreases; in the stage of exhaustion it decreases again (Selye, '50). The decreased adrenal cholesterol content of the rats pair-fed with thiamine-deficient rats (severe food restriction), the markedly increased cholesterol concentration in the rats pair-fed with pantothenic acid-deficient rats (moderate food restriction) and the slightly increased cholesterol content in rats pair-fed with biotin-deficient rats (slight food restriction) might find an explanation on the above mentioned basis. In this connection it is noteworthy that while the deficient animals had adrenal cholesterol of normal specific activity, the pair-fed rats of all groups showed a lower specific activity than the normal controls. In pantothenic acid-deficient rats a much lower adrenal cholesterol content was found, thus confirming earlier cytological evidence of a depletion of lipid material from the zona fasciculata in this nutritional disorder (Ralli and Graef, '44; Deane and McKibbin, '46; Melampy, Cheng and Northrop, '51).

SUMMARY

Thiamine, pantothenic acid, biotin, and choline deficiency were produced in young male rats. Experimental animals with ad libitum and pair-fed controls were given radioactive carboxyl-labeled acetate in their food over a period of three days. At the end of this time the animals were killed and their livers examined for fatty acids and cholesterol, and their heart, adrenals, and blood serum analyzed for cholesterol. C^{14} incorporation in fatty acids and in cholesterol was determined.

In none of the nutritional disorders studied here was any effect of the deficiency upon the specific activity of liver fatty acids and liver, heart, and serum cholesterol noted. Total incorporation of radioactive carbon in the liver fatty acids and cholesterol varied as a result of changes in the absolute amount of these lipids in liver.

The specific activity of adrenal cholesterol was found to be decreased in all types of deficiencies and also in the pair-fed controls.

Pair-fed controls behaved like their respective deficient groups except with respect to total liver fatty acids and activity in the choline-deficient series.

These results support the view that lipogenesis per se is not specifically altered in these deficiencies.

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⁴ Given erroneously in original published title as "pathogenic."

BASAL METABOLISM OF THE ESKIMO

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It has been claimed by several workers that there are distinct racial differences in basal metabolism (Wilson, '45). No satisfactory explanation has been offered for these differences. In some races, such as Eskimos and American Indians, basal metabolic rates (BMR), significantly higher than the figures considered normal for Whites, have been consistently observed. In the case of Eskimos, figures between 13% and 33% over the DuBois standard have been reported by the greater majority of previous investigators (Heinbecker, '28; Rabinowitch and Smith, '36; Crile and Quiring, '39; Höygaard, '41; Bollerud et al., '50).

In connection with studies on human adaptation to cold which were in progress at the Arctic Aeromedical Laboratory, it was considered desirable to examine, under carefully controlled conditions, whether there is any difference in the basal heat production in Eskimos as compared with Whites, and if so, how such a difference may be explained. On this basis, a series of comprehensive studies of basal metabolism has been carried out from 1950 to 1952 on a large number of Eskimos from various native settlements in Alaska. The purpose of this paper is to report briefly the main results of these studies.

MATERIAL AND METHODS

A total of 340 basal metabolism tests were made on 73 healthy Eskimos (52 males and 21 females) from 4 different locations in Alaska, representing different climatic conditions,

living habits and diets. The first group at Barter Island, which is located on the north coast of Alaska, live on a diet consisting of approximately 50% sea mammals and fish, and 50% land mammals. The second group at Anaktuvuk Pass, located in the middle of the Brooks Range 3,000 feet above sea level, live almost exclusively on land mammals, and especially caribou meat. The third group at Kotzebue, situated on the west coast of Alaska, live to a considerable extent on White man's food, and their living habits are more affected by civilization than any of the other groups. The 4th group at Gambell, St. Lawrence Island, live almost exclusively on sea mammals, and especially walrus meat. Of these groups, the Anaktuvuk Pass group had the most limited access to the White man's food.

These 4 groups were examined both in the winter and in the summer. In addition to the field studies, representative subjects of each group have been studied under carefully controlled conditions in our laboratory at Ladd Field while living on the White man's diet. These studies have included environmental surveys, measurements of body surface area, nutritional surveys with particular reference to protein intake, and urinary nitrogen elimination. In some cases, the respiratory quotient was determined.

The subjects, between 20-40 years old, were carefully selected on the basis of complete medical examination, including medical histories, physical examination with x-rays of the chest and long bones, as well as urine and blood examinations. The body weight had been checked several weeks prior to the test to insure that no significant loss of weight had occurred which might influence the results. A total of 28 normal Whites were used as controls.

At each of the 4 Eskimo settlements a complete and well-equipped field laboratory was established, with adequate sleeping quarters for the Eskimo subjects. The standard technique was applied, and all basal metabolism tests were made with the same Benedict-Roth metabolism apparatus. It was tested regularly for leaks, and great care was taken to make the

subjects accustomed to the apparatus prior to the test. Each test consisted of two 9-minute periods. The technique was checked repeatedly both in the field and in the laboratory. We were fortunate in having Dr. E. F. DuBois as a consultant on these studies, and in one case (E. F. D. B.) we were able to compare our results with those obtained from the same subject in the calorimeter at the Russell Sage Institute of Pathology. Our figures came within 1% of the average for the respiration calorimeter. As a further check on our methods, the author's basal metabolism was examined repeatedly, both in our laboratory at Ladd Air Force Base, and in the field. The results in the field were all within 1% of his average.

The subjects were usually examined in groups of 5 on three successive days. Following the last meal at 5:00 or 6:00 o'clock in the evening, the subjects were admitted to the sleeping quarters at the field laboratory, where they spent the night in the presence of a technician in order to ensure reliable control of the fasting. The following morning the basal metabolism tests were commenced at 7:00 A.M., following 30 minutes' rest in the basal metabolism bed after the body weight and height had been recorded. The number of hours' fasting, as well as the oral temperature, pulse rate, and blood pressure were recorded prior to the test. A 5-hour urine sample, starting at 7:00 A.M. was collected for the determination of nitrogen elimination. The greatest possible care was taken in handling of the subjects to achieve maximum relaxation.

It is hardly fair to compare the metabolism of Eskimos on high protein diets with White people on comparatively low protein diets, and perhaps one would be justified in speaking of an "Eskimo basal condition." In most of the previous studies the Eskimo subjects apparently were not basal, and our subjects were not strictly basal until the third day on the White man's diet. However, since we have determined the metabolism 14-18 hours after the last meal, this would

normally fulfill the requirement for "standard metabolism," "post absorptive metabolism" or "basal metabolism."

RESULTS AND DISCUSSION

In agreement with previous workers, it was found that the basal metabolism of Eskimos in their native habitat was significantly higher than in Whites, when examined for the first time while living on their own native diet. The average of the first test of all Eskimos of both sexes from all 4 settlements was 16% higher than the DuBois standard, and the women showed higher figures than the men. The highest figures were observed among the primitive Eskimos at Gambell and Anaktuvuk Pass, and the lowest figures obtained among the more civilized Eskimos at Kotzebue, where the average basal metabolism was only 5% higher than the DuBois standard. In 28 White men, 20-40 years old, examined for the first time, the BMR averaged only 2% below the DuBois standard, i.e., 18% lower than that of the Eskimos.

After having verified the results of previous investigators, the next problem was to explain the reason for this higher basal heat production in the Eskimo.

Of the various factors involved, apprehension would be expected to play an important part in the high basal metabolic rates observed in Eskimos when tested for the first time. It is quite difficult, however, to determine whether or not an Eskimo is apprehensive. The pulse rate and blood pressure cannot always be taken as an indication of the state of relaxation in the Eskimo, since his blood pressure and pulse rate normally are considerably lower than those of the Whites.

During our initial experiments, we observed in several cases a reduction of over 20% in the BMR when the same subject was tested repeatedly on successive days while living on the same diet, although the lowest BMR was still higher than in the White controls. The original high level was interpreted as evidence of apprehension. In some of the subjects, the basal metabolic rate has been determined on as many as 12 differ-

ent occasions, and we find that the drop in BMR generally levels out by the third test.

When comparing the basal metabolic rates in all of the Eskimo subjects as determined on the basis of the first test with the final test, when the lower level had been established on the third and subsequent days, we find an average reduction of 9% which is ascribed to the reduction in tension. This relative reduction was higher in women than in men, and was most pronounced in the most primitive groups.

It was observed that the Eskimo groups that had the highest basal metabolic rates also had the highest protein intake, and

TABLE 1

Average basal metabolic rate, protein intake and urinary nitrogen elimination in Eskimos

LOCATION	BASAL METABOLIC RATE		PROTEIN INTAKE	FASTING URINARY NITROGEN
	Cal./m ² /hour	Deviation from DuBois standard		
		%	gm/day	gm/hour
Anaktuvuk Pass	46.3	+ 16	202	0.9
Gambell	42.9	+ 10	132	0.8
Barter Island	39.7	+ 2	129	0.6
Kotzebue	40.2	+ 3	98	0.6

when comparing the BMR with the urinary nitrogen elimination, the same positive correlation was observed. The highest BMR's and nitrogen eliminations were observed at Anaktuvuk Pass among the caribou meat eaters where the fasting urinary nitrogen at times was as high as 3 gm per hour. The relation between the average BMR, the average daily protein intake at the 4 Eskimo settlements, and the urinary nitrogen elimination determined during a 5-hour period, is shown in table 1.

It was also observed that the basal metabolism was higher in the winter when the protein intake was increased, than in the summer. At Gambell, for instance, the average daily protein intake, as well as the urinary nitrogen elimination, was 20%

higher in March than it was in August. In March the basal metabolic rate was 11% higher than the DuBois standard, while in August it was only 6% over the DuBois standard.

The relation between the high basal metabolism in the Eskimo and the diet has been suggested long ago by several workers, such as Heinbecker ('31), and later Höygaard ('41). Heinbecker studied the basal metabolism of Eskimos at Baffin Island in 1926 and again in 1930. During his first visit he found abnormally high figures for the basal metabolism associated with a high nitrogen metabolism, while during his second visit the basal metabolism was more than 20% lower, and the nitrogen metabolism was markedly reduced.

On the other hand, Odin ('37), who had observed that while basal metabolism was below DuBois standard in the Whites from Norrbotten in Northern Sweden, found that a significant rise in the basal metabolism occurred after an addition of 120-150 gm protein daily in the diet. Similar observations have been made by McClellan and collaborators ('31) and others.

It is well known that considerably higher amounts of protein are regularly consumed by the Eskimos (DuBois, '28), who generally speaking, prefer a diet where approximately 50% of the calories come from protein and the greater part of the remaining 50% are derived from fat. August and Marie Krogh ('13) report that the normal diet of the West Greenland Eskimos contained an excessive amount of animal protein — 280 gm daily — and they noted that there seemed to be a considerable delay in the metabolism of protein and excretion of nitrogen, only 60% of the nitrogen being excreted during the first 24 hours after eating large meals rich in protein. In East Greenland the Eskimos consume an average of 300 gm of protein daily (Høygaard, '41). In Alaska a daily protein consumption of more than 300 gm has been observed among the most primitive Eskimos.

In order to study the significance of the specific dynamic action of the protein in the high meat diet, with reference to the high basal metabolic rates in Eskimos, a special series

of studies was carried out on representative subjects from each of the 4 groups. These subjects, 14 men in all, were first studied in the field on their normal native diet, and then brought into our laboratory at Ladd Field where the basal metabolism again was tested under carefully controlled conditions on three or more different days while the subjects were eating the normal White man's diet. We then found that in all cases the basal metabolic rates were reduced to the range

TABLE 2

Basal metabolic rates in per cent of DuBois standard in 14 Eskimo men

LOCATION	SUBJECT NO.	ON NATIVE DIET	ON WHITE MAN'S DIET
		%	%
Gambell	1	+ 11	— 6
	2	+ 1	— 3
	3	+ 1	— 9
	4	+ 10	— 3
Barter Island	5	+ 9	— 10
	6	+ 14	— 4
	7	+ 13	— 4
	8	+ 6	— 14
Kotzebue	9	— 16	— 14
	10	+ 8	— 2
	11	+ 7	— 15
	12	— 6	— 13
Anaktuvuk Pass	13	+ 14	— 1
	14	+ 12	— 9
Average		+ 8	— 8

of the normal White controls eating the same diet and examined under similar conditions (table 2). At the same time, the urinary nitrogen elimination was reduced to the same level as that of the White controls. Thus, when examined in their native habitat, the average BMR on the third and subsequent days of all the 14 Eskimo subjects was 8% higher than the DuBois standard, and the average urinary nitrogen was 1.2 gm per hour in these Eskimos. The average BMR in the same Eskimos when living on the White man's diet at Ladd

Field was 8% lower than the DuBois standard, and the urinary nitrogen was reduced to 0.4 gm per hour. This change occurred at the end of three days. In a group of 4 normal White men of similar age examined on three successive days under similar conditions and on the same diet, the final BMR was the same as in the Eskimos: 8% lower than the DuBois standard, and their urinary nitrogen elimination was the same as that of the Eskimos (table 3). Similar findings were made in another group of 12 White men examined later. It may be added that normal White controls examined by Dr. E. F. DuBois in New York (personal communication) excreted about

TABLE 3

Average basal metabolism, protein intake and urinary nitrogen elimination of normal white controls compared with Eskimos on White man's diet

SUBJECTS	BASAL METABOLIC RATE		PROTEIN INTAKE	FASTING URINARY NITROGEN
	Cal./m ² /hour	Deviation from DuBois standard		
		%	gm/day	gm/hour
Normal White controls (♂)				
First test	40.4	— 2	75	0.4
Second test	38.9	— 6	70	0.5
Third test	37.8	— 8	60	0.4
Eskimos (♂) on White man's diet	37.4	— 8	73	0.4

0.5 gm urinary nitrogen per hour 14 to 18 hours after the last meal. It should also be noted that the so-called DuBois standards are generally recognized as being 6 to 8% too high, and that the original level, published in 1916, included the factor of tension in patients undergoing their first or second test.

In a separate experiment, the Gambell group was given a high meat diet, and their basal metabolism increased to 12% over the DuBois standard. A similar experiment, using normal White subjects on a high meat diet similar to that of the Eskimos, failed because of the fact that it was impossible for the subjects to become accustomed to eating such

great quantities of meat within the short time available for the experiment. In a later experiment, however, a group of 5 normal White men consumed 500 gm of meat daily (137 gm protein or 21.9 gm nitrogen), and an increase of 13% in the BMR was observed.

It is interesting to note that an Eskimo soldier who had lived for several months on the normal mess hall diet, had a basal metabolic rate 4% lower than the DuBois standard. It should also be noted that the above reported findings were essentially the same in both the 67 full-blooded Eskimos and in the 6 half-breeds examined.

Twenty R.Q. determinations were made in a group of 5 Eskimo subjects living on their native diet. The average R.Q. was 0.82, which is the figure upon which the calculations are based.

It has previously been suggested that the DuBois height-weight formula, when utilized for the calculation of body surface area, may not be applicable for the Eskimo, thus introducing an error in the calculation of the results, which might partly explain the high basal metabolic rates of the Eskimo. The body surface area was determined by the linear method in 53 of our Eskimo subjects, and it was found that the height-weight formula on an average gave results which were 0.9% higher than the results obtained by the linear method. This is within the limit of accuracy claimed for the height-weight chart (DuBois and DuBois, '16) and is therefore insignificant.

SUMMARY AND CONCLUSIONS

Three hundred and forty basal metabolism tests have been made on 73 healthy Eskimos from 4 different locations in Alaska, together with environmental observations, body surface area measurements, and determination of protein intake and nitrogen elimination. In agreement with previous workers, the basal metabolism of Eskimos examined for the first time in their native habitat was significantly higher than in the Whites. Approximately 9% of this higher basal metabolism may be accounted for by apprehension, and the high protein

Eskimo diet accounts for approximately 15%. When these two factors were eliminated, the metabolism was almost exactly the same as in the White controls.

From these studies it is therefore concluded that there are no racial differences between the Eskimos and the Whites in basal heat production.

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FATE OF LYCOPENE IN THE RAT AND ITS EFFECTS ON THE UTILIZATION OF CAROTENE AND VITAMIN A ¹

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The effects of some structurally related compounds on the utilization of carotene and vitamin A have been reported previously (High and Day, '51). Due to the similarity between lycopene and carotene and the rather frequent occurrence of these substances together in nature, it was of interest to investigate the effects of lycopene on the utilization of carotene and vitamin A. Aside from experiments concerned with lycopene being a possible provitamin A (Drummond et al., '25) there are scarcely any data on the metabolic effects and the fate of the substance in the rat.

MATERIALS AND METHODS

The procedure employed for the preparation of lycopene was substantially the same as that described by Sandoval and Zechmeister ('49) except that purification by chromatography was omitted. Microscopic examination of the crystalline prod-

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uct revealed an apparently homogeneous material consisting of long red needles. In hexane the absorption maxima were 445, 472, and 503 m μ and the absorption minima were 455 and 490 m μ . These data agree with both the absorption maxima and minima for pure lycopene in hexane (Zechmeister et al., '43). The molar extinction coefficient in hexane at 472-3 m μ was 16.9×10^4 and, employing the highest molar extinction coefficient reported of 18.6×10^4 (Zechmeister et al., '43), the product on analysis proved to be well over 90% pure lycopene.

Beta-carotene,³ vitamin A,⁴ and lycopene were dissolved separately in cottonseed (Wesson) oil in substantially the same manner as described by High and Day ('51). The depletion of vitamin A stores and the supplementation of the rats were accomplished in essentially the same manner as previously indicated (Kelley and Day, '50). All of the supplements were kept separate and stored under nitrogen in low actinic flasks at 4°C. and each was administered separately in 0.2 ml of cottonseed oil per rat per day.

Approximately 16 hours after the administration of the last supplement, each rat was sacrificed with ether. The livers and kidneys were removed promptly and analyzed immediately for both lycopene and vitamin A. Because lycopene was not detected in the kidneys, analysis of this organ for vitamin A was carried out as previously described (Kelley and Day, '48), by the colorimetric method of Sobel and Werbin ('46). The procedure for analysis of the liver for both vitamin A and lycopene was adapted from the method of Sobel and Werbin ('45), and the method of Dann and Evelyn ('38) was employed for carotene interference in the determination of vitamin A.

The livers were homogenized, saponified, and extracted with ether (Kelley and Day, '48). After removing the ether

³ Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio, consisting of 85% β - and 15% α -carotene.

⁴ Containing approximately 200,000 U.S.P. units per gram. Kindly supplied by Distillation Products, Inc., Rochester, N. Y.

under vacuum the residues were dissolved separately in chloroform and initially analyzed for lycopene with a Klett-Summerson colorimeter employing a 440 m μ filter. The quantity of lycopene present was determined by reference to a standard curve for chloroform solutions of lycopene. Finally, these solutions were analyzed for vitamin A by adding separately 2 ml aliquots of each solution to 4 ml of activated glycerol dichlorohydrin and measuring the intensities of the colors developed at 6 minutes with a Klett-Summerson colorimeter employing a 540 m μ filter. The readings which were obtained represented both lycopene and vitamin A. The increments of these values due to the quantity of lycopene, as initially determined, were ascertained by reference to a standard curve for lycopene and the reagent. These increments were subtracted from the totals and the values for the differences were converted into micrograms of vitamin A by reference to a standard curve for vitamin A and the reagent.

When measured by means of a 540-m μ filter, the colors produced with both lycopene and the reagent, and vitamin A and the reagent, were additive and moderately stable at 6 minutes after mixing. Under these conditions 1.00 μ g of lycopene was equivalent to approximately 0.14 μ g of vitamin A. This is approximately the same relationship that exists between beta-carotene and vitamin A with activated glycerol dichlorohydrin as the chromogenic agent (Sobel and Werbin, '45).

RESULTS

Effects of lycopene in different amounts on the utilization of carotene and vitamin A

Two series of experiments were conducted to determine the effects of different amounts of lycopene on tissue deposition of vitamin A from supplements of carotene or vitamin A. In one series, each rat in a group received 30 μ g of carotene dissolved in 0.2 ml of cottonseed oil per day for 12 days. A second group was treated similarly except that in addition

to the carotene, each rat was given 25 μ g of lycopene per day. In a third group the amount of lycopene was 500 μ g per rat per day. This was in addition to the carotene. The results are summarized in table 1. Twenty-five micrograms of lycopene significantly increased the hepatic storage of vitamin A by 53% while 500 μ g were ineffective. The lycopene seemed to have no effect on the deposition of vitamin A in the kidneys or on growth.

TABLE 1

Effects of different amounts of lycopene on the utilization of carotene and vitamin A

DAILY SUPPLEMENT ¹	NO. AND SEX OF RATS	WEIGHT GAIN	VITAMIN A DEPOSITED		
			Liver	Kidneys	Total
		gm	μ g	μ g	μ g
Series 1					
30 μ g carotene	8(2M)	26	18.4 \pm 2.8	5.9	24.3 \pm 3.0 ²
30 μ g carotene + 25 μ g lycopene	8(2M)	26	28.2 \pm 3.6	6.5	34.8 \pm 2.4
30 μ g carotene + 500 μ g lycopene	10(3M)	23	19.1 \pm 2.8	8.9	28.0 \pm 2.7
Series 2					
13 μ g vitamin A	7(5M)	42	18.5 \pm 1.4	9.5	28.0 \pm 1.4
13 μ g vitamin A + 25 μ g lycopene	7(5M)	40	37.6 \pm 7.9	10.1	47.7 \pm 6.7
13 μ g vitamin A + 100 μ g lycopene	6(4M)	50	32.4 \pm 6.5	8.3	40.7 \pm 4.7
13 μ g vitamin A + 500 μ g lycopene	7(5M)	46	32.6 \pm 7.4	13.3	45.9 \pm 6.6

¹ Fed in cottonseed (Wesson) oil for 12 days.

² The standard error of the mean = $\sqrt{\frac{\sum(X-\bar{X})^2}{n(n-1)}}$ where X = individual values, \bar{X} = the group mean, and n = the number of rats per group.

In another series (table 1) 13 μ g of vitamin A were administered per rat per day in place of carotene. Rats that received 25, 100, or 500 μ g of lycopene per day exhibited increases in hepatic storage of vitamin A as compared with the controls. However, there appeared to be no significant differences between the various levels of lycopene. The average increase in vitamin A deposition for the three groups

was 85%. No differences occurred between either the growth rate or the kidney vitamin A deposition in this series.

Hepatic lycopene deposition

The pigment in the liver of those rats that were fed lycopene with either carotene or vitamin A possessed absorption maxima in chloroform solutions of 457, 481, and 516 m μ . (Chloroform solutions of the unfed lycopene had similar absorption maxima.) According to Strain ('38), lycopene dissolved in chloroform possesses absorption maxima of 453, 480, and 517 m μ . Liver tissues of rats that received either carotene or vitamin A alone possessed negligible absorption in this region of the spectrum. Similarly, spectrophotometric analysis at 480 m μ for the quantity of lycopene present in the liver agreed with the colorimetric determination. From these observations, it seemed logical to conclude that the pigment present in the liver was lycopene. In no instance was the substance detected in the kidneys.

In order to gain information relative to factors influencing lycopene deposition in the liver, data were sought in regard to the deposition of the carotenoid as related to growth rate and to the quantity of lycopene fed. The animals that received 100 μ g of lycopene with either carotene or vitamin A were divided into three groups on the basis of differences in weight gains. Both the lycopene deposition and the weight gain values were averaged for each group. The data are summarized in table 2. They indicated that the amount of lycopene deposited is inversely proportional to the rate of growth.

Data summarized in table 1 (series 2) show the relationship between the amount of lycopene ingested and the quantity deposited in the liver. Although the amount of lycopene deposited was higher with increased intake of the carotenoid, the efficiency of storage decreased with increased intake. For example, rats that received 25 μ g per day for 12 days deposited in the liver 6% of the total amount ingested, while those that received 100 and 500 μ g deposited 3.9 and 2.1%,

respectively. As much as 2.2 mg of lycopene were found in the livers of rats that received 2 mg of the carotenoid per day for 60 days. These data suggest that rats possess a large capacity for the storage of lycopene in the liver.

TABLE 2

Relationship between growth and quantity of lycopene fed and the hepatic deposition of lycopene

SERIES	NO. AND SEX OF RATS	LYCOPENE FED DAILY ¹	AVERAGE WEIGHT GAIN	AVERAGE LYCOPENE DEPOSITED
		μg	gm	μg
1	6F	100	23	61.8
	4F	100	38	51.1
	6(5M)	100	56	36.1
2	8(1M)	25	31	21.1
	9(1M)	100	31	54.7
	9(1M)	500	22	148.0

¹ Fed in cottonseed (Wesson) oil for 14 days.

DISCUSSION

The sparing effect of small amounts of lycopene on carotene and vitamin A may be due to protection of the provitamin and vitamin from oxidative decomposition in the alimentary tract as previously suggested for the effects of small amounts of lutein on carotene utilization (Sherman, '47; High and Day, '51). Its apparent failure to exert a protective effect on carotene when the amount ingested is large may be due to a type of competitive inhibition of the conversion of carotene to vitamin A. Thus, in the presence of large amounts of lycopene the sparing effect may be masked by an impairment in the formation of vitamin A. Some clarification of this problem may be achieved when it becomes possible to effect the enzymatic conversion of carotene to vitamin A in a strictly *in vitro* system.

Prior to this investigation scarcely any experiments had been reported concerning the fate of lycopene fed to rats. However, a few observations had been made on other species of animals. Gillam and Kon ('40) found no evidence of lyco-

pene absorption in cows. On the other hand, the carotenoid has been found in varying amounts in human abdominal fat, liver, and serum (Zechmeister and Tuzon, '35; Willstaedt and Lindquist, '36).

In contrast, the rat does not absorb appreciable amounts of either lutein or carotene as such. The factors responsible for this selective absorption of these carotenoids are obscure. Both lutein and carotene possess the ionone ring structure and lycopene is devoid of it. Possibly this difference in structure accounts for the difference in absorption.

SUMMARY

Relatively small amounts of lycopene fed with either carotene or vitamin A to vitamin A-depleted rats increased the utilization of both of the latter substances for tissue deposition of vitamin A. Large amounts of lycopene increased vitamin A deposition in vitamin A-fed rats but were without effect on vitamin A deposition from carotene. Lycopene was absorbed and deposited in the livers of rats in approximately 2-6% of the total amount ingested. No lycopene was detected in the kidneys.

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EVALUATION OF NITROGEN SOURCE MATERIALS BY AN INTRAPERITONEAL RAT TEST ¹

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ONE FIGURE

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The biological value of a nitrogen source material is an index of the efficiency with which it meets the nitrogen needs of an animal. Methods of estimating the biological value of protein hydrolysates and of amino acid mixtures intended for intravenous use in states of protein depletion currently are receiving a great deal of attention both by interested laboratories and by such regulatory agencies as the Food and Drug Administration and the Pharmacopoeial authorities. Methods are vitally needed for distinguishing between preparations which have little or no biological value, and those which have been formulated so as to meet adequately the needs of the organism.

Two methods currently being studied for official adoption are based either on intravenous administration into dogs (Frost and Risser, '46; Kade, Houston, Krauel and Sahyun, '46; Silber and Porter, '49) or on oral ingestion by rats (Frazier, Wissler, Steffee, Woolridge and Cannon, '47; Frost and Sandy, '48). The former procedure is not practical for routine use since it is time consuming and costly. Rat feeding methods should be objected to because they do not simulate the conditions of use, since they permit the intestinal enzymes to digest any contained peptides in a manner which

¹ Presented in part before the American Institute of Nutrition, Cleveland, April 30, 1951 (Arnold and Shepherd, '51).

does not take place after intravenous injection. As an example, the availability of the peptide nitrogen after oral ingestion by dogs of one preparation was indicated by Silber and Porter ('49) as the reason for its good biological value, although it had a value of zero when administered intravenously. Obviously, such a preparation would be of little value to a patient being fed by vein.

A third method, suggested by Silber and Porter ('50), depends upon determining the nitrogen retention of rats given a preparation parenterally at a single dosage level over a one- to 4-day period. Quantitative results in terms of biological value are obtained by the method of Allison and Anderson ('45), the endogenous nitrogen excretion being estimated by including a depletion control group.

In the present study it has been shown that when the test is set up at multiple levels of intake, the indicated nitrogen retention is markedly different from that estimated by Silber and Porter ('50) on the basis of a single level of intake and a depletion control group. Therefore, the results from a single dosage level experiment may not yield correct values. However, those based on multiple dosage levels obviate the need for a depletion control group, and are a valid index of the biological value of the test preparation. Data illustrating these points are given in the following paragraphs. Furthermore, if the method presented herein is generally adopted, it will afford a rapid and convenient method of measuring the biological value of intravenous protein injection mixtures, and will lend confidence to the selection of appropriate solutions for clinical use. In addition to the description of the method for evaluating nitrogen source materials given in the following sections, the results of applying the method to preparations currently employed clinically and to gelatin are included.

EXPERIMENTAL

Test materials

Defatted whole eggs. Vacuum packed dried whole eggs were extracted with hexane in a two-liter capacity Soxhlet extractor

for three days. The solvent was removed from the extracted eggs by allowing them to remain in thin layers on paper overnight.

Tryptophan-deficient hydrolysate. A tryptophan-free hydrolysate was prepared for these studies by Dr. A. Lesuk of this Institute who hydrolyzed casein with dilute sulfuric acid. In the absence of reducing agents, this procedure destroys tryptophan. The hydrolysate, after the removal of the acid with barium in the usual way and supplementation with small amounts of methionine and phenylalanine to compensate for losses during the treatments, was concentrated to a 6% solution of the hydrolyzed protein. The solution was sterilized for use and was supplied in 1 l portions.

Gelatin. To determine the effect of peptide-bound nitrogen under these conditions of test, a gelatin preparation was administered both orally and parenterally. The preparation used was a 6% one identified by the Knox No. P 48-20. It had not been prepared to serve as a source of nitrogen so much as for use as a plasma extender.

Commercial hydrolysates and amino acid mixtures. Five commercially available preparations intended for clinical use were evaluated by the proposed procedure. They were obtained on the market.

Diet

The essentially nitrogen-free diet had the following composition (in parts per 100): dextrin 41.88, glucose (cerelose) 41.87, salts (Jones and Foster, '42) 4, methyl cellulose 2, cod liver oil (2,000 units of vitamin A, 250 units vitamin D per gram) 1, hydrogenated vegetable oil (Primex) 9, choline chloride 0.15, inositol 0.1; (in milligrams per 100 gm) calcium pantothenate 2, riboflavin 0.6, niacinamide 0.4, pyridoxine hydrochloride 0.4, folacin 0.2, *p*-aminobenzoic acid 0.2, thiamine hydrochloride 0.2, menadione 0.02, biotin 0.02, and vitamin B₁₂ 0.002.

Procedure

Female rats of 200 to 300 gm body weight were caged individually. To reduce their nitrogen stores they were fed the nitrogen-free diet ad libitum for a week. They were then fed weighed amounts of the diet each day over a 4-day period. The amount fed was one that all of the rats given any one nitrogen supplement would voluntarily consume, e.g., 8 gm per 200 gm rat per day. The diet was fed each morning before the supplements were administered.

The test materials were administered intraperitoneally using sterile precautions with needles of about 24 gauge and syringes of 5 to 15 ml capacity varying with the amount of solution given. The site of injection on the rats was swabbed with 70% alcohol prior to injection. In addition, to reduce the risk of infection, about 200,000 units of penicillin per liter were added to the solutions.

The animals were allowed one day for equilibration. Then, at the time they received their second injection, the rats were placed in metabolism cages. Aside from rinsing the sides of the cages with a little water each day the urine and feces samples required no other attention until they were collected at the end of the three-day experimental period. The possibility of nitrogen loss was minimized by adding a crystal of thymol and a few drops of sulfuric acid to the urine collecting bottles at the time they were placed under the funnels. The feces, kept separate from the urine by having been caught on glass wool at the neck of the metabolism rack funnels, were collected and placed in 1:1 sulfuric acid for stirring prior to taking aliquots for analysis.

Analyses

Analyses for nitrogen were made by the macro-Kjeldahl method, using copper as a catalyst. The food and feces nitrogen samples were digested for 6 hours after clearing and the urine samples for one and one-half hours.

TABLE 1

Biological value of defatted whole egg fed to one-week protein depleted adult rats and of a tryptophan-free hydrolysate administered intraperitoneally

TEST SUBSTANCE	NUMBER OF RATS	AVERAGE WEIGHT		NITROGEN INTAKE <i>mg/kg</i>	NITROGEN EXCRETION		NITROGEN "BALANCE"		BIOLOGICAL VALUE
		At depletion	Final		Urinary	Fecal	N intake — urinary N	N intake — (UN+FN)	
Test no. 16 None	3	238	219	...	192	94	...	— 286	
	5	234	232	145	182	95	— 37	— 132	
	6	230	218	200	179	106	+ 21	— 85	
	5	223	214	289	176	113	+ 113	0	0.92
Test no. 17 None	4	254	236	...	171	73			1.04
	6	252	236	135	169	89	— 34	— 123	
	6	252	242	181	175	82	+ 6	— 76	
	6	252	244	255	173	81	+ 82	+ 1	0.98
Test no. 18 Tryptophan- free hydrolysate	6	284	270	149	205			— 56	1.03
	5	283	271	207	271	64		— 64	
	6	284	269	299	355			— 56	0.0

TABLE 2
Biological value of a 6% gelatin solution (Knox no. P 48-20) given orally or intraperitoneally

ROUTE OF ADMINISTRATION	NO. OF RATS	AVERAGE WEIGHT		NITROGEN INTAKE		NITROGEN EXCRETION		NITROGEN BALANCE		BIOLOGICAL VALUE	
		gm	gm	mg/kg	mg/kg	Urinary	Fecal	UN only	UN + FN	UN only	UN + FN
Orally	6	238	227	226	177			mg/kg			
	5	240	229	321	272			48			
	5	239	232	461	337			48		0.15 ±	
	6	234	227	682	577			124		0.06	
Intra- peritoneally	6	216	206	76	201		62	105			
	6	216	205	150	266		52	— 125	— 187		
	6	216	207	298	386		56	— 116	— 168	0.18 ±	0.19
								— 88	— 144	0.06	

Calculation of results

The evaluations are based upon the slope of the regression line calculated from the data which show the nitrogen balance at different levels of nitrogen intake. The slope of this line is an expression of the rate of change of nitrogen balance with respect to nitrogen intake and is a valid measure of the biological value of the protein. It has been used by Bricker and Mitchell ('47) for evaluating proteins ingested by rats

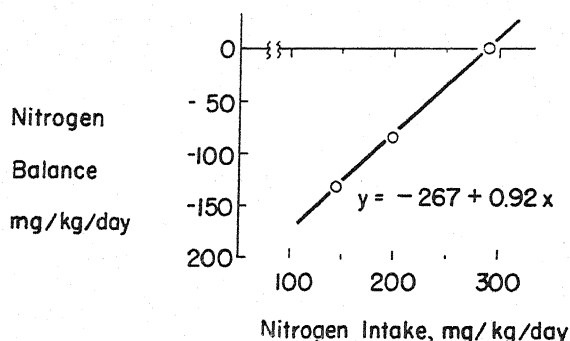


Fig. 1 Graphical representation of increase in nitrogen balance (retention) of rats fed defatted whole egg protein in increasing amounts.

orally, and by Melnick and Cowgill ('37) and Allison and Anderson ('45) for dogs given nitrogen source materials orally and parenterally.

The constant 'a' of the regression equation $y = a + bx$ (Waugh, '43) is the calculated nitrogen balance at zero nitrogen intake. For defatted whole egg of test no. 16 given in table 1 the value calculated for 'a' is -267 mg nitrogen per kilogram per day. It may be compared with the experimentally determined value of -286 mg nitrogen per kilogram per day given in table 1. The constant 'b' of the regression equation is the slope of the line. The data for defatted whole egg of table 1 are given in figure 1 to demonstrate representative results graphically.

RESULTS AND DISCUSSION

Defatted whole egg protein. It may be seen from the data summarized in table 1 that the nitrogen of defatted whole egg was essentially completely retained and the biological value of the protein was very close to 1.0. Since the defatted whole egg protein was fed along with the diet, the results do not bear upon the matter of parenteral feeding. It appears reasonable to conclude, however, that the data offer supporting evidence for the view that, aside from the matter of the parenteral administration of nitrogen source materials, which is discussed below, the over-all procedure might be expected to yield valid conclusions.

A separate point which may be seen from the feeding of defatted whole egg protein is that under conditions of controlled food intake the fecal nitrogens are relatively constant. This being the case essentially the same biological value is indicated from the calculations based upon urinary nitrogen alone or upon both urinary and fecal nitrogen excretions.

Tryptophan-deficient hydrolysate. The effect of administering a poor nitrogen source material upon nitrogen retention is presented in the data obtained by administering a tryptophan-free hydrolysate, as summarized in table 1. There was increased nitrogen excretion with increasing nitrogen administration so that the nitrogen retention did not increase. In accordance with the expected result, the biological value of the material was accordingly zero.

However, if the slope of response, e.g., the biological value, had been based upon the nitrogen excretion at zero nitrogen intake (171 to 192 mg/kg, table 1) and any one of the balance values observed for the tryptophan-free hydrolysate (— 56 to — 64 mg/kg), some positive value would have been ascribed to the preparation, which would have been erroneous. It is clear that misleading conclusions may be drawn from single level tests.

An instance of this in the literature may be used to emphasize the point. Silber and Porter ('50) ascribed positive biological value to amino acid mixtures deficient in one or another of the essential amino acids. Their calculations were based upon one of their test levels along with the nitrogen excretion at zero nitrogen intake. However, if the latter value is not used, and if their data are recalculated on the basis of the nitrogen balances at the two levels of administration, the evaluations from their data are in accord with the expected results, namely, amino acid mixtures deficient in one of the essential amino acids do not support nitrogen retention at increasing levels of nitrogen administration. Stated in another way, their preparations had biological values of essentially zero. Results such as these, in addition to our own observations given above, support the validity of conclusions based upon multiple level tests.

Gelatin. Because of the possible interference of peptide-bound nitrogen in test materials evaluated by the proposed procedure, a preliminary check on this effect was made by giving 6% gelatin (Knox No. P 48-20) orally and parenterally. The results are summarized in table 2. They do not supply any instance of higher nitrogen retention by oral than by parenteral administration, in spite of a probably high proportion of peptides. The value 0.15 to 0.19 for gelatin reported here may be compared with that of 27% nitrogen retention observed in dogs by Rhode, Parkins and Vars ('49) and that of 25% by Mitchell, Beadles and Kruger ('27). Rhode et al. ('49) noted considerable individual variation, which is also indicated in these studies by the high standard error of the results.

The 4-level oral assay of gelatin relates to the matter of straight line responses. Without discussing the data in detail, it is apparent from the nitrogen balance data of table 2 that so much variation existed that almost any value for gelatin could have resulted if less than 4 levels of intake had been given depending on which, and how many, of the points had been omitted. Whether the reason for the wide variance

in data yielded in the tests on gelatin is other than its high content of peptides is not evident to us. In any event, gelatin does not meet the biological value criterion of 0.40, i.e., 40% nitrogen retention, suggested by Mitchell ('52) as the minimum necessary to support growth in some species. Mitchell's suggested criterion of biological value is met by each of the commercial hydrolysates discussed below, which clinical experience has shown to be useful. Thus, Mitchell's further suggestion that "biologic evaluation of proteins and protein mixtures is still the 'court of last resort' " is fully supported by the findings submitted here.

As a separate point, it may be seen from the data in tables 1 and 2 that the rats lost weight during the test. This was due to the fact that the voluntary food intake of the animals decreased during the depletion period to a point where they would not voluntarily consume their caloric needs during the test (repletion) period. The loss in weight did not vary widely for the animals administered any one preparation and the results did not depart from linearity to a significant extent. This may have been due to the short period of time of the test period; continuing weight loss over a longer period of time might have exerted a discernible unfavorable effect. We have not studied this point, however.

Commercial hydrolysates and amino acid mixtures. The results of applying the proposed procedure to commercially available nitrogen source materials intended for parenteral use are summarized in table 3. It may be seen that the preparations have biological values in the range of 0.44 to 0.63, when the evaluations are based on total nitrogen excretion. The values obtained by this procedure are in the same range as those reported by Silber and Porter ('49) for comparable preparations evaluated by parenteral administration in dogs. Tested orally in dogs the same investigators noted that the nitrogen of the preparations was retained somewhat better, 61 to 73%.

It is seen that all of the preparations tested here were well utilized. This is in contrast to the report of Silber and Por-

ter ('49). These investigators noted a preparation (C) which was well utilized when given orally ($66 \pm 2.0\%$ nitrogen retention) but which was not utilized at all parenterally ($0 \pm 13\%$). Presumably, this occurred because the digestive enzymes rendered available one or more peptide-bound essential

TABLE 3

Biological value of commercially available nitrogen source materials intended for intravenous alimentation evaluated by the multiple level parenteral rat test

PREPARATION	TEST NO.	NUMBER OF RATS	NUMBER OF LEVELS OF TEST	BIOLOGICAL VALUE BASED ON	
				UN + FN	UN only ¹
A	21	12	3	0.495	0.61 (± 0.09) $r = 0.91$ ²
	33	18	2	0.51	0.58
B	22	13	3	0.63	0.56 (± 0.12) $r = 0.82$
	25	12	3	0.57	0.54 (± 0.09) $r = 0.89$
C	24	14	4	0.50	0.51 (± 0.07) $r = 0.90$
	23	14	3	0.48	0.53 (± 0.15) $r = 0.71$
D	25	10	3	0.62	0.62 (± 0.11) $r = 0.90$
	27	15	3	0.49	0.52 (± 0.11) $r = 0.80$
E	27	15	3	0.44	0.47 (± 0.11) $r = 0.96$
	28	15	2	0.48	0.49 (± 0.08)

¹ Along with the biological value is given its estimated standard error where (Snedecor, '46) $s_b = \sqrt{(\bar{S}y^2 - (Sxy)^2/Sx^2)/(n-2) (Sx^2)}$.

² The correlation coefficient, r , was calculated from the Waugh ('43) formula $r = Sxy/(Sx^2) (Sy^2)$.

amino acids which were not available when the preparation was given parenterally and were not acted upon by the digestive proteolytic enzymes. The question of the effect of the route of administration thus does not appear to arise when the proposed procedure is applied.

Several additional points of importance may be discussed on the basis of the data supplied from the study of the clinical preparations. More often than not, the evaluation based solely on urinary nitrogen excretion agrees with the evaluation based on total excretion, but the instances of disagreement which do occur indicate that misleading conclusions might be drawn from time to time if the fecal nitrogen output is not taken into account. Thus, we cannot recommend that determination of the fecal nitrogen excretion be omitted.

It has been our practice to examine the individual urines in preference to a pooled sample in order to obtain some indication of the error of procedure. The fecal excretions take more time to prepare so that on these we have only analyzed the pooled samples. Therefore, the balance values based on urinary plus fecal nitrogen excretions are not characterized by an estimate of the error (s_b).

Also, it may be noted from the data in table 3 that the preparations have been tested at two, three, or 4 levels of intake. The r (correlation coefficient) values indicate that the data satisfy the statistical criteria for a straight line response. This correlation coefficient is meaningful only when the preparations are tested at three or more levels. Having established this point, a two-level test with the nitrogen intakes widely spaced, e.g., 100 and 300 mg/kg, favors reducing the error of the estimation (Smith, '51).

At this time, on the basis of the data submitted above, the proposed procedure thus appears to offer a valid method of evaluating nitrogen source materials parenterally in a simple manner.

SUMMARY

A method is described whereby nitrogen source materials intended for intravenous alimentation may be evaluated by intraperitoneal administration to adult rats, which have been protein depleted for one week. In its simplest form, the preparations are administered at two levels for 4 days, excreta collections being made during the last three days. The excreta may be pooled for analysis.

Evaluation of a 6% gelatin solution by both parenteral and oral test indicated it to have about the same biological value, 0.15 to 0.19, thus leaving open the question as to the nutritive value of the contained peptide-bound amino acids.

Five commercial preparations of known clinical usefulness when parenterally administered were observed to have biological values in the range of 0.44 to 0.63.

The data show conclusively that evaluations based upon multiple levels of test may be materially different from those based upon single level tests in conjunction with the nitrogen excretion at zero nitrogen intake. The use of multiple levels thus appears to be mandatory for valid assays.

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NITROGEN BALANCE OF ADULT RATS FED DIETS LOW IN L- AND DL-LYSINE, OR DEVOID OF ARGININE¹

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ONE FIGURE

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The indispensability of lysine for rat growth has been firmly established. Most investigators are convinced that lysine is also indispensable for the maintenance of nitrogen or weight equilibrium in the adult animal. Wolf and Corley ('39) found that omission of any one of the essential amino acids, except arginine, from the diet of adult rats resulted in negative nitrogen balance and weight loss. Frazier et al. ('47) also observed weight losses under similar experimental conditions. The adverse effect of withdrawal of lysine on nitrogen balance was confirmed by Wissler et al. ('48). Gillespie et al. ('45) reported that rats deprived of dietary lysine developed a mild anemia. They suggested, however, that this response was not specific for lysine deficiency and that it might occur in the absence of any of the other essential amino acids. Rose and Rice ('39) demonstrated that the adult dog requires dietary lysine for the maintenance of nitrogen equilibrium. The minimum quantity of lysine required by the adult rat for the maintenance of body weight was estimated by Neuberger and Webster ('45) to be 16 mg/day/rat (three animals with average body weight of 335 gm),

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and by Steffee et al. ('50) to be 60 mg/day/kg (150-gm rat). Benditt et al. ('50) reported that the minimum daily requirement of lysine for maintenance of nitrogen equilibrium in the adult male rat is 3.7 mg/day/100 cm² of body surface.

Burroughs, Burroughs and Mitchell ('40) concluded that the adult rat does not need lysine, leucine, histidine, phenylalanine or arginine for the replacement of endogenous losses of nitrogen if threonine, isoleucine, tryptophan, valine, methionine, tyrosine and norleucine are present in the diet. Mitchell ('47) reiterated this point of view with respect to lysine and concluded that "... lysine is entirely dispensable in adult rodent nutrition, or is required in inconspicuous proportions for the maintenance of nitrogen equilibrium."

The experiments on lysine described below represent a portion of a larger project which eventually will provide quantitative data on the requirement of the adult rat, under uniform conditions, for all of the essential amino acids.

METHODS

Adult male albino rats of the Wistar strain were used. At the beginning, and after a 48-hour fast with water ad libitum, the body weights ranged from 210 gm to 254 gm (mean = 232 gm). The energy allowance for each rat was 40.4 Cal./day. Many observations in this laboratory make it evident that energy and weight balance are satisfactorily maintained if 121 Cal./day are allowed for each unit of metabolic body size, i.e., Cal./rat/day = 121 kg^{3/4}. Each experiment included the following sequence of feedings: maintenance diet (9.6% whole egg protein), two weeks; nitrogen-free (N-free) diet, one week; amino acid diet, containing approximately half of the maintenance requirement of total nitrogen (half-N), one week; amino acid diet, containing twice this amount of total nitrogen (full-N), one week. This 5-week cycle of feeding was repeated for each amino acid mixture investigated.

The amino acid mixture simulates the proteins of whole egg in that it contains approximately the same amount of each natural isomer of the essential amino acids per gram

of total nitrogen. The non-essential amino acids of egg protein are replaced, in this mixture, by the unnatural isomers of 6 essential amino acids plus sufficient L-glutamic acid to make up the same amount of total nitrogen. This "complete" amino acid mixture and the basal diet in which it is used are described elsewhere (Anderson and Nasset, '48; Nasset, Anderson and Siliciano, '51).

The N-free diet was compounded simply by omitting the amino acid mixture and substituting an equal weight of sucrose.

It is the custom in this laboratory to observe changes in nitrogen balance which are brought about by a stepwise reduction of the concentration of one essential amino acid at a time. This process simulates the consecutive testing of a series of proteins of different biological values. It is probably a much more physiological procedure than omitting completely one of the essential amino acids from the mixture. At some point in this stepwise reduction a concentration is reached which becomes limiting for the utilization of the total dietary nitrogen. In the present investigation two-fifths and one-twenty-fifth L-lysine and one-twelfth DL-lysine amino acid mixtures were used. In addition, one mixture was tested in which arginine was absent. The fractions refer to the total amounts of lysine present, regardless of isomeric form, as compared with the "complete" amino acid mixture. One-twelfth DL-lysine, for example, simply refers to a mixture which contains one-twelfth as much lysine nitrogen as the complete mixture and identifies the source as the racemic form.

The N-free, half-N and full-N diets were fed by stomach tube in two equal portions daily. The maintenance diet was weighed out accurately each day in feeding cups to provide each animal with a constant intake of protein and energy. This portion was almost invariably eagerly and completely consumed. Total N was determined on feed and excreta by the Kjeldahl method. Each diet period lasted 7 days and feces were collected for the entire period. They were marked

by adding Cr_2O_3 to the first feeding of each period. The urine was collected for analysis only during the last 4 days of each period of amino acid feeding. Other details of experimental procedure are described in an earlier paper (Nasset and Anderson, '51).

RESULTS

Table 1 contains the average results of all experiments with the rats of series 320. The discovery of a limiting concentration of the essential amino acid being studied may require considerable preliminary work. The first guess is usually made on the basis of data taken from the literature but this technique invariably yields estimates which are too high. Inspection of the results of experiment II shows at once that the nitrogen balance, the value of K' and the total nitrogen computed to be necessary for the maintenance of nitrogen equilibrium (NI_e) are unaffected by reducing the L-lysine content of the diet to two-fifths of the lysine content of the complete diet (experiments I, V and VI). According to these three criteria the amino acid mixture used in experiment II is nutritionally equal to the one used in experiment I. According to the same criteria the one-twenty-fifth L-lysine amino acid mixture is significantly poorer than the ones used in experiments I and II and here the small amount of L-lysine available is affecting adversely the utilization of the dietary nitrogen. Fortunately then, a limiting concentration of L-lysine which was not too severely deficient was discovered on the second trial.

The responses to feeding the complete amino acid mixture, as well as those in which the amino acid being investigated has not been critically reduced (experiments I, II, V and VI), confirm many previous experiments in this laboratory. A conspicuous feature is the absence of any change in either fecal or urinary N excretion when the intake of N is doubled. This fact indicates complete utilization of the increment in N intake and speaks well for the adequacy of the complete amino acid mixture for the maintenance of N equilibrium

TABLE 1
Average data for adult rats receiving amino acid diets

EXPERIMENT NUMBER	SERIES 320					
	I	II	III	IV	V	VI
AMINO ACID MIXTURE	Complete	2/5 L- Lysine	1/25 L- Lysine	1/12 DL- Lysine	Complete	Complete minus arginine
Number of rats	11	14	13	10	11	10
Average body weight (kg)	0.240	0.246	0.244	0.253	0.261	0.269
Metabolic body size (kg ^{3/4})	0.343	0.349	0.347	0.357	0.365	0.374
Milligrams of nitrogen per day per kg ^{3/4}						
Half N-period:						
N intake	70	71	72	67	69	65
Fecal N	37	34	33	31	30	28
Urinary N	109	108	121	101	102	105
N balance	-76	-72	-82	-66	-63	-68
Full N-period:						
N intake	140	136	141	132	132	129
Fecal N	34	31	31	29	26	25
Urinary N	109	110	146	134	112	105
N balance	-4	-5	-35	-31	-7	-2
K' (half-N; full-N) ¹	1.03 ± 0.06	1.01 ± 0.04	0.67 ± 0.03	0.53 ± 0.04	0.90 ± 0.03	1.06 ± 0.06
NI _e ²	141 ± 4	141 ± 2	195 ± 4	194 ± 7	140 ± 3	130 ± 3

¹ K' is the slope of the line joining the half-N and full-N points when nitrogen balance is plotted against nitrogen intake.

² NI_e is the nitrogen intake computed to be necessary for attainment of nitrogen equilibrium.

under these experimental conditions. For the reader who is unaccustomed to the unit of metabolic body size ($\text{kg}^{3/4}$), it may be helpful to state that the total N intake of the average 250-gm rat on a full-N diet was approximately 50 mg/day.

DISCUSSION

The results in table 1 reveal that a rather drastic reduction in lysine intake is required to produce an adverse effect on nitrogen balance in the adult animal. In experiment II (two-fifths L-lysine) the lysine was reduced to 40% of the amount in the complete amino acid mixture (experiments I and V) without causing any detectable change in nitrogen balance. In fact, the values for nitrogen balance, K' and NI_e , are virtually identical in experiments I and II. When the concentration of lysine is reduced to 4% (one-twenty-fifth L-lysine, experiment III), however, the changes, when compared with experiment II, are significant throughout. Nitrogen balance is depressed at both levels of intake ($P < 0.03$ and < 0.0001 for half-N and full-N, respectively), K' is reduced ($P < 0.0001$) and the amount of nitrogen computed to be required for nitrogen equilibrium, NI_e , is increased ($P < 0.0001$).

The preponderance of evidence supports the view that D-lysine is not utilized by the animal for either growth or maintenance. McGinty et al. ('24) reported that racemic lysine was only half as effective as the natural isomer in promoting growth of rats on a diet containing gliadin as the source of protein. Berg ('36) observed that the growing rat is unable to utilize D-lysine as a supplement to zein and Totter and Berg ('39) made a similar observation on the young mouse. Kratzer ('50) demonstrated that turkey poults are unable to utilize D-lysine for either growth or feather pigmentation. Rose ('49) reported, without supporting data, that D-lysine cannot be utilized by the adult human male. Albanese ('47) stated, on the basis of unpublished results, that more than 80% of orally administered D-lysine is utilized by man.

The results of the present investigation show conclusively that the adult rat is unable to utilize D-lysine in the mainte-

nance of nitrogen balance. A comparison of the results of experiments III and IV (table 1) shows clearly that one-twelfth DL-lysine is approximately equivalent to one-twenty-fifth L-lysine with respect to nitrogen balance, K' and NI_e . Such a result would be expected if the unnatural component of the racemic mixture were inactive.

Melnick and Cowgill ('37) and Bricker et al. ('45) demonstrated in dogs and humans, respectively, that nitrogen balance is a linear function of protein intake, especially when nitrogen balance is negative. In previous work with valine, methionine and threonine (Nasset and Anderson, '50, '51; Nasset, Anderson and Siliciano, '51) it was demonstrated

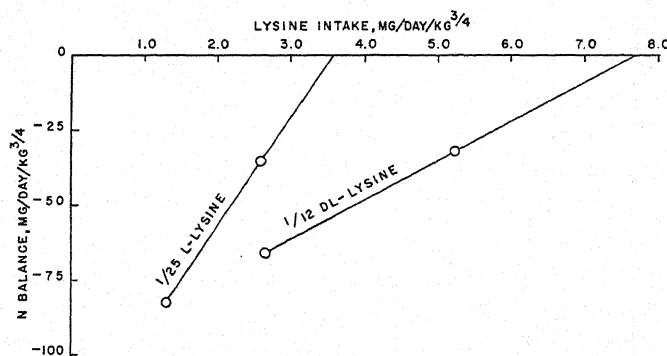


Fig. 1 Nitrogen balance plotted against lysine intake.

that nitrogen balance is a linear function of the intake of the limiting essential amino acid. This fact was used to compute the daily requirement of these amino acids for the maintenance of N equilibrium.

In figure 1 nitrogen balance is plotted against lysine intake for the two experiments in which this amino acid was present in limiting concentrations (experiments III and IV, table 1). An extrapolation upward of the line joining the two average points obtained from each of these experiments yields graphic estimates of the lysine required for maintenance of N equilibrium. A more satisfactory estimate is obtained by extrapolating the lines joining the individual points

obtained for each rat for each experiment. The mean values thus obtained, expressed as milligrams of lysine/day/kg^{3/4} required for nitrogen equilibrium are: L-lysine = 3.6 ± 0.07 and DL-lysine = 7.8 ± 0.27 . The results demonstrate clearly that D-lysine is not utilized under these conditions. Using the methods of Nasset and Siliciano ('52), the results of Benditt et al. ('50) and Rose ('49), for the adult rat and human, respectively, were computed on the same basis. The requirement of lysine as determined by these investigators is roughly 10 times as great as the value reported here. Benditt et al. ('50) used very few animals in their work and this may account for their relatively high value. An obvious reason for the high value given by Rose is the fact that what he reports as a minimum requirement is not a mean of his minima but actually his highest minimum among the group of subjects who ingested any particular amino acid.

The evidence presented for the indispensability of lysine in the nutrition of the adult rat appears to be unequivocal. The requirement is very small under these conditions, i.e., 3.6 mg lysine/day/kg^{3/4}, or approximately 1.25 mg/day/250-gm rat. This fact may account for Mitchell's ('47) conclusion that lysine is dispensable for the adult rat. He fed 4 rats 10 gm/day of a diet in which the protein was chiefly derived from white flour and the total daily nitrogen intake for each rat was 67.6 mg. If it is assumed that all of the nitrogen came from white flour, it can be computed from the data of Block and Bolling ('51) that Mitchell's rats may have been ingesting approximately 8 mg of lysine per day. Since this is several times the minimum requirement, as established in the present investigation, it is to be expected that supplementation of white flour with lysine would fail to improve the nitrogen balance.

SUMMARY

Nitrogen balance was determined on a group of adult male albino rats which derived all of their dietary nitrogen from mixtures of amino acids. Each experiment included the following dietary regimens in the order given: 14 days on mainte-

nance diet (9.6% whole egg protein); 7 days on N-free diet; 7 days on amino acid diet supplying approximately half of the maintenance requirement of total nitrogen; 7 days on double the quantity of the amino acid mixture fed in the previous period. These diets, except the maintenance diet, were fed by stomach tube in two equal portions daily, and each rat received the same amount of diet each day.

If the quantity of lysine in a complete mixture of essential amino acids is reduced sufficiently, the nitrogen balance is adversely affected. When this amino acid is the limiting factor in the utilization of dietary nitrogen, it is assumed that N balance is a linear function of lysine intake. On this assumption the requirement for the maintenance of N equilibrium is 3.6 ± 0.07 mg of L- and 7.8 ± 0.27 mg of DL-lysine/day/kg^{3/4}.

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EVALUATION OF THE BIOLOGICAL VALUES OF THE PROTEINS IN FISH MEALS BY THE NITROGEN RETENTION METHOD¹

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A review of the literature shows the need of detailed information on the biological values of the proteins in fish. Deuel and associates ('46) found that mixed mackerel muscle proteins have a considerably higher biological value than casein. Tuna proteins when fed at 9 and 12% and sardine proteins at 15% also gave considerably more rapid growth in weanling rats than diets containing similar amounts of casein.

Since fish meals, which are by-products of the fish industry, are being used extensively as sources of proteins in animal feeding, it was thought of interest to investigate their biological values. With the cooperation of the By-Products Division of the National Fisheries Institute, Washington, D. C., we were supplied with the following products from various parts of the country: sardine meal; Alaska herring meal; herring meal with fish solubles, which is from a different source than the Alaska herring meal; Red fish meal; Menhaden fish meal; anchovies meal; and crab meal. The fish meals were furnished by the processors in 50-lb. lots selected from several batches; hence the products received constituted representative samples.

The biological values of the proteins in the fish meals were determined by the nitrogen balance method of Mitchell ('24,

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'44), in which the values are expressed as the per cent of absorbed nitrogen retained by the animal. From the biological values and digestibility figures the per cent of net utilization was calculated. Wistar strain albino rats were used and in all of the groups the sexes were divided equally. Each group contained 12 animals. The animals were about 28 days old when started on the experiments and they weighed 50 to 56 gm each. In order to eliminate the influence of the plane of nutrition, we used controlled feeding. Each group was fed the fish meals at an 8% protein level; each animal was given 8 gm daily of its ration which was totally consumed by every rat in the group; therefore, the food intake was constant. Each ration contained an amount of fish meal to provide 8% protein. The rest of the rations contained percentagely cellu flour for roughage, 2; Sure's salts No. 1 ('41), 4; vegetable shortening, 8; cod liver oil, 2; wheat germ oil, 1; and the balance, cerelose. The fat-soluble vitamins A, D, and E were furnished by the cod liver oil and wheat germ oils. The following components of the vitamin B complex were administered separately from the ration 6 times weekly: 25 μ g of thiamine, riboflavin, pyridoxine, and niacin, respectively; 150 μ g calcium pantothenate, 1 mg inositol, 3 mg *p*-aminobenzoic acid, and 9 mg choline chloride.

In order to have information on the biological values of the proteins in fish meals expressed in comparison with efficiency of proteins of known excellent quality, nitrogen retention studies were also carried out on dried whole eggs and on dried non-fat milk solids.

Urinary and fecal balances were carried out for 7 days on an egg standardizing ration and also for 7 days on the experimental rations containing the various fish meals. In each case the animals were allowed to become accustomed to consuming the entire rations for a pre-test period of from three to 5 days before the beginning of the nitrogen balance studies. The percentage composition of the egg ration is as follows: dried defatted whole egg, 5.8; cellu flour, 2.0; Sure's salts No. 1, 4; vegetable shortening, 8.0; cod liver oil, 2; wheat

germ oil, 1; and cerelese, 77.2. The defatted dried whole eggs contained 69.2% protein and furnished 4.0% protein in the ration.

The results of this study are summarized in tables 1 and 2. It will be noted from table 1 that the protein content of the fish meals varied from 35.0 to 69.7%; the fat from 2.1 to 15.3%; the ash from 11 to 29.3%; and the moisture from 2.3 to 8.4%. By varying the amounts of fish meals in the rations it was possible to adjust the protein content to 8% but, because of the great variations in fat content, the rations could not be adjusted on an isocaloric basis; also, because of the great variations in ash, the total mineral content of the rations

TABLE 1
Proximate chemical analyses of fish meals

FISH MEALS	PROTEIN	FAT	ASH	MOISTURE
	%	%	%	%
Alaska herring meal	69.7	8.4	11.0	6.0
Herring meal with fish solubles	52.1	15.3	15.4	8.4
Sardine meal	58.4	3.3	21.8	2.3
Menhaden fish meal	63.4	15.0	15.9	3.3
Red fish meal	57.1	8.1	25.3	6.0
Anchovies meal	50.3	3.3	26.1	6.0
Crab meal	35.0	2.1	29.3	7.0

was also different. However, it is evident from table 2 that the high mineral content of Red fish meal did not interfere with its excellent protein utilization; therefore, the lower net utilization of protein in the crab meal is not due to its high mineral content but rather to its low digestibility. The large standard deviations in the biological value and digestibility of crab meal are due to individual differences in nitrogen retention and digestibility. The standard deviations for digestibilities for Red fish meal and the herring meals seem high, but they are not any higher than that of dried non-fat milk solids. The least standard deviations for biological values and digestibilities were found for the proteins in dried whole eggs.

TABLE 2

The relative biological values of the proteins of various fish meals, dried whole eggs, and dried non-fat milk solids fed at an 8% protein level. Daily food intake, 8 gm. Daily nitrogen intake, 102.4 mg

SOURCE OF PROTEIN	BODY WEIGHT	FECAL N ¹	MET. ² N IN FECES	FOOD N IN FECES	ABSORBED N	N IN URINE	MET. N IN URINE	FOOD N IN URINE	FOOD N RETAINED	TRUE DIGESTIBILITY	BIOLOGICAL VALUE ³	NET UTILIZATION ⁴
	gm	mg	mg	mg	mg	mg	mg	mg	%	%		
Sardine meal	72.5	14.9	12.7	2.2	100.2	33.3	19.7	13.0	86.6	97.8	86.4	81.5
Menhaden fish meal	66.5	21.1	10.1	11.0	91.4	33.5	21.5	12.0	79.4	89.2	86.9	77.5
Red fish meal	67.3	23.7	10.2	13.5	88.9	33.8	22.5	11.3	77.6	86.8	87.3	75.8
Alaska herring meal	76.7	20.8	7.7	13.1	89.3	36.9	18.8	18.1	71.2	87.2	79.7	69.5
Herring meal with fish solubles	68.6	20.6	10.0	10.6	91.8	38.7	22.4	16.3	75.5	89.6	82.2	73.6
Anchovies meal	66.6	35.0	11.0	24.0	78.4	35.1	21.5	13.6	64.8	76.6	82.6	63.1
Crab meal	65.9	40.7	10.7	30.0	72.4	32.4	24.4	8.0	64.2	70.7	85.9	60.2
Dried non-fat milk solids	68.9	21.0	10.4	10.6	91.8	30.3	23.1	7.2	84.6	89.6	92.2	82.6
Dried whole eggs	78.8	13.3	11.2	2.1	100.3	25.5	22.0	3.5	96.8	97.9	96.5	94.5

¹ N signifies Nitrogen.

² Met. signifies Metabolic.

³ Per cent of absorbed nitrogen retained in the body.

⁴ The value for the true coefficient of digestibility multiplied by the biological value divided by 100.

It is apparent from table 2 that the proteins of dried whole eggs rank first in efficiency, followed by those of dried non-fat milk solids. However, all of the fish meals have high biological values. The lower net protein utilization of crab meal and anchovies is due to their low true digestibilities. When the net utilization of the proteins in dried whole eggs is taken as 100, those of dried non-fat milk solids are 88.4, and the various fish meals are calculated as follows: sardine meal, 89.4; Menhaden fish meal, 82.0; Red fish meal, 80.2; Alaska herring meal, 73.6; herring meal with fish solubles, 77.9; anchovies meal, 66.7; and crab meal, 63.7.

SUMMARY

A study was made of the biological values of various fish meals by the nitrogen retention method. All of the fish meals were found to have high biological values. However, because of low digestibilities, the net utilization of the proteins in crab meal and in anchovies meal was lower. The biological values were as follows: sardine meal, 86.4; Menhaden fish meal, 86.9; Red fish meal, 87.3; Alaska herring meal, 79.7; herring meal with fish solubles, 82.2; anchovies meal, 82.6; and crab meal, 85.9. When the net protein utilization of dried whole eggs is taken as 100, the various fish meals have the following values; sardine meal, 89.4; Menhaden fish meal, 82.0; Red fish meal, 80.2; Alaska herring meal, 73.6; herring meal with fish solubles, 77.9; anchovies meal, 66.7; and crab meal, 63.7.

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The formal presentation will be made at the annual meeting of the Institute in the spring of 1953. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 1, 1953. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

Chairman, Nominating Committee:

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*Department of Biological Chemistry
University of Utah Medical School
Salt Lake City, Utah*

OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of \$1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1953 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 1, 1953.

Chairman, Nominating Committee:

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METHIONINE AND SELENIUM TOXICITY^{1, 2}

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FIVE FIGURES

(Received for publication April 14, 1952)

A high-protein ration affords greater protection against selenium poisoning in animals than one of a low-protein content (Moxon, '37; Smith, '39; Smith and Stohlman, '40; Gortner, '40; Lewis, Schultz and Gortner, '40; Rosenfeld and Beath, '46). Of the commercial proteins, crude casein and linseed meal have shown the greatest protection against liver damage in rats. Linseed meal was the only one to give protection to dogs (Moxon, '41) and to poultry (Anderson, Poley and Moxon, '41). The factor in the proteins responsible for this protection has not been identified. Since methionine has been reported (Heppel, Porterfield and Sharpless, '47) as a protective agent against liver damage by various toxic agents, it was suggested that this protein factor might involve methionine. However, the literature reports concerning methionine and liver damage due to selenium toxicity are conflicting.

Smith ('39) concluded that in selenosis the protein-selenium ratio in a diet was more important than the intake level of selenium, and that methionine supplementation did not help.

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Lewis, Schultz and Gortner ('40) reported that a 30% casein diet gave greater survival and less growth retardation against 25 to 50 p.p.m. of selenite selenium than isocaloric diets containing 6% casein. Methionine supplementation afforded similar protection when added to a 6% casein diet, but cystine did not help. A methionine-supplemented 15% arachin diet also gave greater protection against selenium than plain arachin. Smith and Stohlman ('40) concluded that it was the protein (casein) that was protecting against 15 p.p.m. selenite selenium and not the cystine or methionine supplements. Recently, Fels and Cheldelin ('50) reported a partial alleviation of selenium toxicity in yeast and Sellers, You and Lucas ('50), using a methionine-supplemented diet, found a protection against 20 p.p.m. of selenate selenium in the rat only when a 0.05% tocopherol acetate supplement was included in the diet.

This paper presents evidence to show that methionine does not alleviate selenium poisoning in the rat when seleniferous wheat is fed.

EXPERIMENTAL

The control diet used in all these experiments had the following percentage composition:

Wheat, control, 2.44% N	84.25
Casein, commercial, 12.45% N	10.00
Salts IV (Phillips and Hart, '35)	1.00
Yeast, dried Brewer's 8.27% N	1.00
Lard	3.00
A and D feeding oil	0.75

The substitution of seleniferous wheat (assaying 23 p.p.m. selenium and 2% N) for the control wheat in the above diet was the only difference between the control and toxic diets. The methionine supplemented diets consisted of the designated grams of DL-methionine and a sufficient amount of the proper diet to make 100 gm. The animals were kept in individual cages and were fed and watered ad libitum. Table 1 lists animal groups and diets used in the 5 series.

TABLE 1
Animal group data and identification

ANIMALS AND DIETS	SERIES I	SERIES II	SERIES III	SERIES IV	SERIES V
Initial wt. and sex	210-300 gm females	65 gm males	60 gm males	80 gm males	80 gm males
Number per group	2	4	5	10	5
DIETS					
	GROUPS				
Control	I	I	I	I	I
Control + 2% methionine (M.)	II	II	II		
Control + 1% M.			III		
Control + 0.5% M.					
Control + 0.5% M. + 0.05% tocopherol acetate (T.A.)					
Toxic (19 P.P.M. Se)	III	III	IV	II ¹	II
Toxic + 2% M.	IV	IV			III ¹
Toxic + 1% M.			V		
Toxic + 0.5% M.			VI	III ¹	
Toxic + 0.5% M. + 0.05% T.A.					IV ¹

¹ The selenium content of this diet was 13 p.p.m. This was made by using 56.2% seleniferous wheat and 28.1% control wheat with the other ingredients of the control diet unchanged.

RESULTS

The growth curves of the rats in the first series are shown in figure 1. Although there were considerable individual variations in response to the diets, the loss in weight caused by the toxic diet was not prevented by the addition of methionine to the diet.

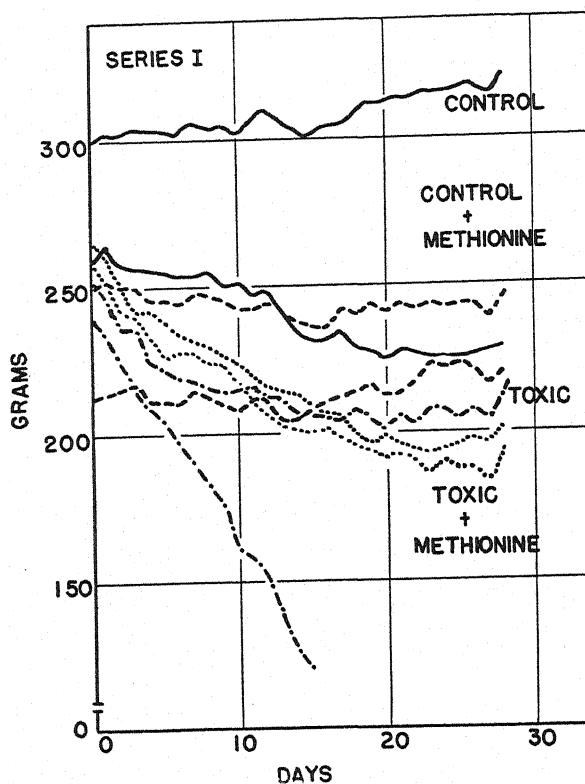


Fig. 1 Growth record of series I.

The animals of series II were young rats that gained steadily in body weight when fed the control diet. As shown in figure 2, the 2% level of methionine inhibited growth. The animals on the seleniferous diet showed the characteristic growth depression with the first death occurring on the 19th day. The group fed the selenium plus methionine diet gained

slightly more, but two of the 4 rats died. The other two were heavier, probably due to some accumulation of ascitic fluid.

The data of figure 3 show that levels of 1.0% and 0.5% methionine do not appreciably affect the growth rate of rats re-

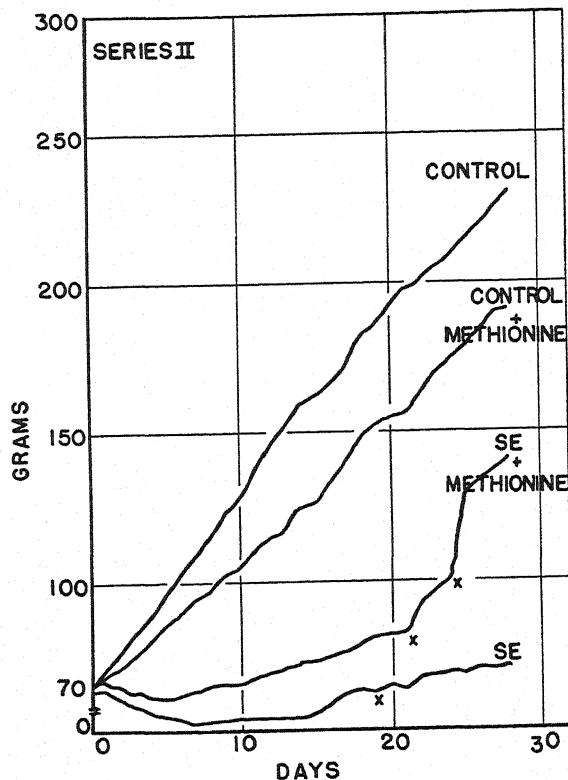


Fig. 2 Growth record of series II. X = death of an animal.

ceiving the control diet. Growth was poor and mortality was high in all groups receiving selenium. In the selenium group without added methionine, only one rat lived to the end of the trial (46 days). All others that received the toxic diets were dead by the 35th day.

Older rats and a lower selenium content in the toxic diets than in the third series were used in series IV. The data

in figure 4 show a slightly better weight gain from the selenium plus methionine diet than from the selenium diet alone, but the early deaths and extensive liver damage previously associated with the former group still occurred. On analysis, the livers from the animals receiving the toxic diet averaged 8.4 p.p.m. of selenium and of the selenium plus methionine

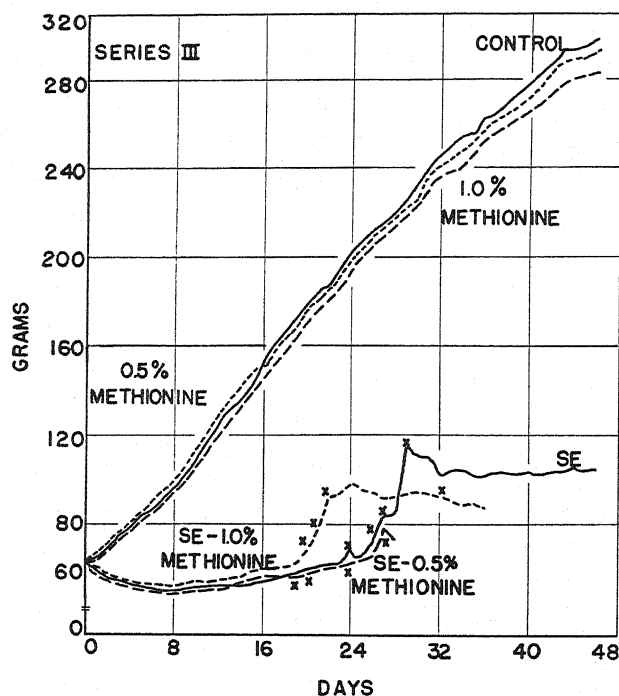


Fig. 3 Growth record of series III. X = death of an animal.

diet, 10.1 p.p.m. Thus, methionine supplementation of the diet had no beneficial effect on reducing the selenium contents of livers.

Further lack of protection by methionine even when supplemented with 0.05% α -tocopherol is shown in figure 5. Four of the 5 animals receiving selenium plus methionine plus tocopherol died during the 5th week of the experiment, whereas three of the selenium fed rats were alive at the termination

of the experiment. A better growth rate was observed in the former group, but autopsies showed severely damaged livers and other characteristic symptoms of selenium poisoning. The results show that supplements of methionine and tocopherol do not prevent the tissue damage caused by naturally occurring selenium.

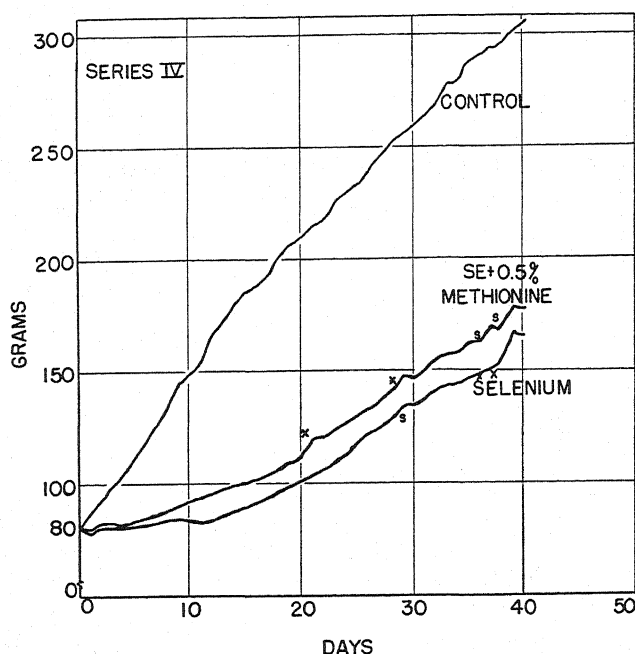


Fig. 4 Growth record of series IV. X = death of animal. S = sacrificed.

The average food consumption for each of the 5 series showed that the presence of methionine in the control diet did not change the food intake nor did it alter the lowered food consumption commonly associated with the ingestion of a seleniferous diet. There was no significant variation in the water intake among the various groups.

The methionine and protein contents of the diet are shown in table 2. The data for methionine have been calculated from Block and Bolling ('45) because selenium interfered in the

analysis of the seleniferous wheat for methionine by the method of McCarthy and Sullivan ('41). The methionine requirement for the rat as given in the literature varies from 0.5% (Treadwell, '48) to 1.2% (Womack and Rose, '41)

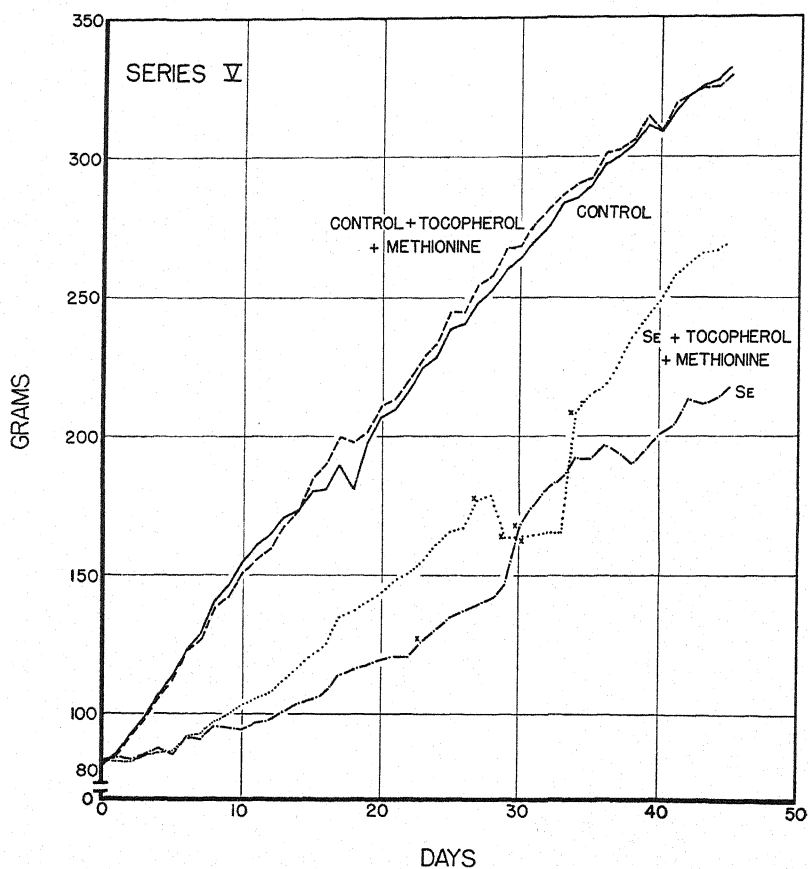


Fig. 5 Growth record of series V. X = death of an animal.

of the diet. The animals of series IV and V that received the selenium diet ate approximately 8 gm of diet or 67 mg of methionine daily. The animals receiving the control diets ate about 15 gm or 114 mg of methionine daily. These diets were not deficient in sulfur-containing amino acids and even

TABLE 2
Methionine and protein contents of diets

COMPONENT	AMOUNT	DIETARY PROTEIN	METHIONINE CONTENT OF MATERIAL		METHIONINE FURNISHED BY MATERIAL PER 100 GM DIET
			Calc.	Found	
			%	%	
Control wheat	84.25	11.1	0.64	0.67	0.539
Casain	10.00	7.9	2.77	2.88	0.277
Yeast	1.00	0.5	1.04	1.00	0.010
Toxic wheat	84.25	9.6	0.52	selenium interferes	0.438
Totals for control diet	95.25 ¹	19.5			0.826
Totals for toxic diets					
Series I, II, III	95.25	18.0			0.725
Series IV, V	95.25	19.5			0.759

¹ Diets of series I, II, III, had 84.25% seleniferous wheat and no control wheat. Diets of series IV and V had 56.2% seleniferous wheat and 28.05% control wheat. See table 1.

if the methionine were not available in the seleniferous rations, 2% supplements of methionine should have sufficiently enriched the diet to mitigate the selenium toxicity if methionine were effective.

Fels and Cheldelin ('48) described the mechanism of selenate toxicity in yeast as one of a competitive inhibition for an enzyme, necessary for the synthesis of methionine from sulfate. Complete reversal of the selenate toxicity was accomplished with sulfate but never with methionine, and the authors acknowledged that "other processes . . . are concerned." Although sulfate sulfur is significant in animal metabolism (Block, Stekol and Loosli, '51), the role of the sulfur-containing amino acids as a sulfur source for the animal body is especially so and any interference in the metabolic scheme becomes important. The failure of the great excess of methionine sulfur compared with the selenium present to prevent the toxicity refutes the idea of a metabolic antagonism or an *in vivo* inactivation of methionine by selenium.

The selenium level used in the first three series was very toxic in relation to the protein content, but reduction of the selenium content of the diet still did not result in any protection from methionine. The danger of an amino acid imbalance is lessened in this series, but it is doubtful if this is critical.

The detoxification of selenium through the respiratory system, possibly as methyl selenide, could involve methionine as a methyl donor, but Lewis, Schultz and Gortner ('40), and Jacobi and Baumann ('42), have failed to show any relationship between methylating agents and the exhaled selenium-containing gases. The selenium compounds present in these gases have not been characterized to date but work is being done on this phase of selenium poisoning in this laboratory.

The data of this experiment show that methionine does not prevent the toxic action of selenium compounds occurring naturally in feeds, when fed to rats. Further work is in progress with proteins which show protective action.

SUMMARY

Experiments with varying levels of dietary methionine (0.5% to 2%) have shown that this amino acid is ineffective in the protection of rats against the toxicity of selenium occurring naturally in feeds.

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SYNTHESIS OF CERTAIN B-VITAMINS IN THE COBALT DEFICIENT SHEEP, WITH SPECIAL REFERENCE TO VITAMIN B₁₂¹

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Abelson and Darby ('49) indicated that radio-cobalt fed to sheep is incorporated into fecal vitamin B₁₂. Using a chick assay for vitamin B₁₂ in rumen ingesta, Hale and co-workers ('50b) demonstrated that synthesis of this factor was greatly limited in cobalt-deficient sheep as compared to cobalt-fed sheep. Recent work has shown that vitamin B₁₂ is an important intermediary in cobalt metabolism in ruminants (Smith et al., '51; Koch and Smith, '51; Marston, '52; Hoekstra et al., '52). The evidence indicates that cobalt deficiency manifests itself as a vitamin B₁₂ deficiency, and that the cobalt is necessary for incorporation by certain rumen organisms into vitamin B₁₂ active substances which are required by the host animal. It seemed desirable to investigate further the status of the cobalt-deficient sheep as to rumen synthesis, blood level, and liver storage of vitamin B₁₂.

The work of Ray et al. ('47) showed lower blood levels of certain B-vitamins (B₆, niacin, and riboflavin) in the cobalt-deficient sheep. Hale et al. ('50a) reported that a complete B-vitamin supplement exclusive of vitamin B₁₂ reversed the symptoms of a cobalt-deficiency; however, the work of Keener

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et al. ('50) showed no beneficial value of these B-complex vitamins for the cobalt-deficient sheep. Gall and co-workers ('49) have reported that an altered flora occurs in the cobalt-deficient sheep. There existed the possibility of a general inhibition of rumen synthesis in a cobalt deficiency and this was also investigated in relation to the nicotinic acid and riboflavin content of the rumen ingesta of cobalt-deficient sheep.

EXPERIMENTAL

In December of 1950 a group of spring lambs were fed a basal cobalt deficient ration. The basal ration had the following composition: chopped, mixed, grass and alfalfa hay 49.25%; ground oats 49.25%; urea "262" feeding compound 1.50%. The cobalt contents, in parts per million, of these three ingredients, were, respectively, 0.06, 0.03 and 0.07. This ration was fed together with a free choice of iodized salt and tricalcium phosphate. Nine lambs were given supplemental cobalt by mixing one ounce of $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ in each 100 lb. of salt. Three of these 9 lambs were limited in feed to the amount eaten by three animals on the basal ration only. Some of the remaining lambs were initially fed a supplement of 2% dried fish solubles (very low in vitamin B_{12}) or the ash from an equivalent amount of fish solubles. After 6 months it was found that this level of fish solubles, or its ash, did not prevent nor significantly delay the appearance of cobalt deficiency symptoms. These animals were definitely cobalt-deficient and were transferred to the basal ration. Thus, essentially this experiment consisted of three lots of animals: lot 1, which received ad libitum the basal cobalt-deficient ration; lot 2, which received ad libitum the basal cobalt-deficient ration plus supplemental cobalt; and lot 3, another control lot on the basal plus cobalt ration but limited in amount ingested to approximately that of the lot 1 animals.

Blood samples were taken by jugular vein puncture into citrated tubes. They were immediately refrigerated until assays were made. Assays were set up on the same day the blood samples were collected. Aliquots were diluted with

distilled water according to the expected blood vitamin B₁₂ content, and 5 duplicate levels were added to the assay tubes for each sample. This method gave good results for sheep blood.

The livers were obtained at the time of slaughter or death of the animals. In no case was an animal dead for more than two or three hours before the liver was taken. Livers were stored in a frozen state until assays were made. A representative weighed sample of liver was homogenized with water in a Waring Blendor. The homogenized samples were then heated in boiling water for 10–12 minutes, filtered, the protein precipitate washed several times with boiling water, and the filtrate made up to a given volume. Aliquots of this filtrate were diluted according to the concentration of vitamin B₁₂ in the sample. This non-enzymatic method of releasing vitamin B₁₂ from livers gives good liberation according to Scheid et al. ('51a, b) and unpublished data at Wisconsin. To determine the alkali-stable vitamin B₁₂ activity of the livers an aliquot of the original filtrate (representing a 1:200 dilution) was taken to approximately pH 12.5 with 1 N KOH, autoclaved for 30 minutes at 15 lb. pressure, adjusted to the pH of the media (6.5), diluted to give a final dilution of 1:400, and then assayed along with the original liver samples.

Rumen samples were also collected at slaughter. Representative samples of the contents were homogenized with water in a Waring Blendor and stored in a frozen state until assayed. The vitamin B₁₂ of weighed aliquots of these samples was released by incubation with takadiastase and papain at pH 4.5 in acetate buffer. The samples were steamed briefly, adjusted to pH 6.5, filtered, and then diluted to a suitable volume for vitamin B₁₂ assay. Nicotinic acid and riboflavin were also determined on the homogenized samples. Samples of the homogenized rumen ingesta were dried at 100°C. and the vitamin concentrations reported on dry weight.

Vitamin B₁₂ was determined microbiologically using *Lactobacillus leichmannii* 4797 by the method of Thompson et al. ('50). Five-minute sterilization periods at 15 lb. pressure

were used and the response was measured titrimetrically with 0.1N NaOH after a 72-hour incubation period. Five duplicate levels were set up for each sample. The data usually represented averages of two or more separate assays on each sample. Riboflavin was determined with *Lactobacillus casei* using the modified Snell-Strong medium and procedure as described by Snell ('50) following the recommended acid extraction procedure. The collaborative U.S.P.-A.O.A.C. 1945 media and procedure using *Lactobacillus arabinosus* described by Snell ('50) were used to determine nicotinic acid.

RESULTS

The body weight data are presented in tables 1, 2 and 3 to indicate the severity of the cobalt deficiency. There can be no doubt that the animals in lot 1 were suffering from a cobalt deficiency. Table 1 summarizes the blood vitamin B₁₂ concentrations of the three lots of experimental sheep. These data are for a relatively early, but a definite, stage of cobalt deficiency. In later stages of cobalt deficiency, vitamin concentrations in the blood could not be reliably evaluated, since use of too high a concentration of citrated blood inhibited growth of the assay organism. The blood vitamin B₁₂ levels of the cobalt-deficient sheep are only about one-fifth of those encountered in animals on an identical ration supplemented with inorganic cobalt. Considerable variation is noted in the blood vitamin B₁₂ concentrations of cobalt-fed animals, especially in those given limited amounts of feed. The higher average vitamin B₁₂ content of the blood of animals in lot 3 is probably a result of the consumption of larger quantities of salt containing the supplemental cobalt. These animals were being starved, and as a result of intense appetite consumed approximately twice as much salt per head as the animals in lot 2.

Table 2 presents the vitamin B₁₂ data of the livers before and after alkali treatment for the three experimental lots. The difference in the two values gives an approximation of the "true" vitamin B₁₂ activity. Subtraction of the alkali

stable vitamin B₁₂ activity from the total vitamin B₁₂ activity for lots 1 and 2 shows that the livers from animals fed cobalt contained, on the average, about 30 times as much "true" vitamin B₁₂ activity per gram of moist liver as those from cobalt-deficient animals. In the cobalt-deficient lot nearly

TABLE 1

Comparison of blood vitamin B₁₂ values of sheep in early stages of cobalt deficiency with those of cobalt-fed, full-fed sheep and cobalt-fed, limited-fed sheep

LOT	NO. SHEEP	BODY WT. STATUS	VITAMIN B ₁₂ ACTIVITY OF WHOLE BLOOD (MUG/ML) ¹
1 (— Co)	16	Lost ave. of 6.8 lb. of max. body wt.	0.47 ± 0.11
2 (plus Co, full-fed)	6	Gained ave. of 27 lb. for period of 10 wks. prior to bleeding	2.3 ± 0.6
3 (plus Co, limited-fed)	3	Lost ave. of 10 lb. of max. body wt.	4.3 ± 1.5

¹ Mean ± standard deviation.

TABLE 2

Comparison of the vitamin B₁₂ content of livers of cobalt-deficient sheep with that of cobalt-fed, full-fed sheep and cobalt-fed, limited-fed sheep

LOT	NO. SHEEP	BODY WT. STATUS	AVE. FINAL BODY WT.	TOTAL VITAMIN B ₁₂ ACTIVITY (MUG/GM MOIST LIVER)	ALKALI STABLE VITAMIN B ₁₂ ACTIVITY (MUG/GM MOIST LIVER)
			lb.		
1 (— Co)	9	Lost ave. of 45 lb. prior to death or slaughter	65	0.055 ± 0.015	0.025 ± 0.015
2 (plus Co, full-fed)	6	Ave. daily gain of 0.24 lb. throughout exp. period	133	0.93 ± 0.26	0.009 ± 0.006
3 (plus Co, limited-fed)	3	Lost ave. of 18 lb. prior to slaughter	79	1.24 ± 0.20	0.035 ± 0.002

one-half of the vitamin B₁₂ activity was stable to alkali treatment, while only about 1% of the total activity remained in the case of the cobalt-fed, full-fed animals. Lot 1 and especially lot 3 showed a greater amount of alkali stable vitamin B₁₂ activity in liver tissue than lot 2.

Results of rumen ingesta analyses presented in table 3 show a much lower synthesis of vitamin B₁₂ by cobalt-deficient sheep. A high degree of variation was noted in the cobalt-

TABLE 3

Comparison of the vitamin B₁₂, riboflavin and nicotinic acid content of rumen ingesta of cobalt-deficient sheep with that of cobalt-fed, full-fed sheep and cobalt-fed, limited-fed sheep

LOT	NO. SHEEP	BODY WT. STATUS	AVE. FINAL BODY WT.	VITAMIN B ₁₂ ACTIVITY (μG/GM DRY WT.)	NICOTINIC ACID (μG/GM DRY WT.)	RIBOFLAVIN (μG/GM DRY WT.)
			lb.			
1 (— Co)	4	Lost ave. of 34 lb. prior to slaughter	60	0.09 ± 0.06	51 ± 16	10.5 ± 1.1
2 (plus Co, full-fed)	5	Ave. daily gain of 0.26 lb. throughout exp.	143	1.3 ± 0.4	59 ± 5	12.6 ± 0.8
3 (plus Co, limited-fed)	3	Lost ave. of 18 lb. prior to slaughter	79	1.3 ± 0.9	59 ± 4	14.3 ± 2.2

fed lots. This was probably because of the variations in intake of cobalt prior to slaughter. Nicotinic acid values do not differ among the three groups. Although there appears to be a slight inhibition of riboflavin synthesis in the cobalt-deficient lot, the values do not differ enough to represent a serious limitation of riboflavin to the sheep.

The data indicate a much lower rumen synthesis of vitamin B₁₂, also a much lower blood level of vitamin B₁₂, and a severe depletion of liver storage of vitamin B₁₂ in cobalt-deficient sheep as compared to cobalt-fed sheep.

DISCUSSION

Certain reservations must be considered in accepting vitamin B₁₂ values as determined by *L. leichmannii*. It is known that various forms of vitamin B₁₂ have different activities for this organism (Broquist et al., '51). A conversion of vitamin B₁₂ to a more active form on autoclaving has been reported by Broquist et al. ('51); these authors have discussed the significance of this fact in the interpretation of microbiological assay of natural materials when vitamin B₁₂ is used as the standard. The demonstration of the presence of vitamin B_{12f} in rumen ingesta of the sheep by Lewis et al. ('52a, b), and the production of pseudo-vitamins B₁₂ by an isolated rumen anaerobe reported by Pfiffner and co-workers ('51) may tend to overevaluate the vitamin B₁₂ potency of samples from the cobalt-fed animals. It is known that although both of these materials are active for *L. leichmannii*, vitamin B_{12f} is inactive for the rat, and the pseudo-vitamins B₁₂ are inactive for the chick, rat, and man.

Despite these limitations in the determination of vitamin B₁₂, the differences found in this study are so profound that there is little doubt that the cobalt-deficient sheep were severely limited in vitamin B₁₂ as compared to the sheep given supplemental cobalt.

The blood vitamin B₁₂ concentration obtained for the cobalt-deficient sheep was at the lower range of the values reported by Couch and co-workers ('50; 0.6-0.9 µg/ml), while in the cobalt-fed sheep the vitamin B₁₂ blood level was considerably above their range of values. Our data indicate that the concentration of vitamin B₁₂ in blood is dependent on the intake of cobalt, and that literature values for blood of ruminants are subject to this consideration. It should be pointed out that all of the animals in lot 1 were in early stages of cobalt deficiency; the animals' body weight status ranged from a stationary body weight for 6 weeks prior to bleeding to a maximum loss of 23 lb. over a period of 12 weeks prior to bleeding. Meanwhile the cobalt-fed animals were making good body weight gains.

The vitamin B₁₂ concentration of the livers of cobalt-deficient sheep compare with values reported for rat liver (Scheid et al., '51a; Lewis et al., '49), while the cobalt-fed sheep liver values compare more with reported beef liver analyses (Schweigert et al., '51). The vitamin B₁₂ activity of liver after alkali treatment is generally assumed to be due to the desoxyribosides (Hoffman et al., '49). Scheid, Andrews and Schweigert ('51) have shown that a large portion of the vitamin B₁₂ activity of rat liver for *L. leichmannii* is alkali stable. This was also true for the livers of cobalt-deficient sheep in this study. In approximating the "true" vitamin B₁₂ activity by deducting the alkali stable activity from the total activity, it is assumed that the two values are strictly additive. This assumption may or may not be valid. Scheid and Schweigert ('51) have demonstrated a higher alkali stable vitamin B₁₂ activity of liver from rats supplemented with vitamin B₁₂ than from those deficient in vitamin B₁₂. It is not clear why the alkali stable activity was higher in our experiment for the cobalt-deficient sheep livers than for the livers from cobalt-fed, full-fed sheep. It is of interest to note, however, that the livers from the cobalt-fed, limited-fed animals also contained a much higher alkali stable vitamin B₁₂ activity than the livers of the full-fed animals.

The fact that the levels of nicotinic acid and riboflavin per gram of dry rumen material are essentially the same for cobalt-fed and cobalt-deficient sheep suggests that rumen synthesis, at least for these two vitamins, was not impaired by the lack of cobalt. It should be remembered, however, that cobalt-deficient animals are at a much lower nutritional plane and the rumen-fill is much less than in a normal animal. It is not surprising, then, to find the blood levels of certain vitamins lower in these animals.

SUMMARY

Microbiological assays with *Lactobacillus leichmannii* for the vitamin B₁₂ activity of the blood, livers, and rumen ingesta of cobalt-deficient sheep, cobalt-fed, full-fed sheep, and cobalt-

fed, limited-fed sheep have been presented. The blood of sheep early in cobalt deficiency contained only about one-fifth the vitamin B₁₂ activity of the blood from cobalt-fed, full-fed sheep. Liver storage of vitamin B₁₂ was very low in the cobalt-deficient sheep; about 30 times more "true" vitamin B₁₂ activity was present in the livers of cobalt-fed animals. The alkali stable vitamin B₁₂ activity of liver seemed to be higher for the deficient sheep and the cobalt-fed, limited-fed sheep than for the cobalt-fed, full-fed sheep. Rumen synthesis of vitamin B₁₂ in the cobalt-deficient animals was about one-fifteenth of that encountered in the sheep given supplemental cobalt. Microbiological assays indicated that the rumen synthesis of nicotinic acid and riboflavin was essentially the same per gram of dry rumen material in deficient and cobalt-fed animals.

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RESPONSE OF COBALT-DEFICIENT SHEEP TO INTRAVENOUSLY ADMINISTERED VITAMIN B₁₂¹

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THREE FIGURES

While cobalt deficiency symptoms in sheep have been readily alleviated by oral administration of as little as 0.1 mg of cobalt per day, injection of similar or much larger amounts of cobalt have produced at most very slow responses (Becker et al., '49; Keener et al., '50; Marston and Lee, '49; Phillipson and Mitchell, '50; Ray et al., '48). This suggested that the action of cobalt was through the rumen. Keener et al. ('51) have shown that if radioactive cobalt is injected in relatively large amounts, it can pass into the rumen in significant amounts, thereby explaining the slow response of cobalt-deficient sheep to the injection of cobalt.

Filmer ('33) and Filmer and Underwood ('37) recognized the curative effects of liver on cobalt deficiency in sheep. The ash from an equivalent amount of liver would not alleviate the deficiency symptoms. They suggested that the potency of liver was due to the presence of a stored organic factor, and that cobalt functioned through the production of this factor in the body (probably via the rumen). With the discovery that vitamin B₁₂ contained cobalt (Rickes et al., '48; Smith, '48), the immediate postulation was that

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cobalt functioned through incorporation into vitamin B₁₂ in the rumen. Early testing of this hypothesis led several groups of workers to conclude that the physiological role of cobalt in sheep was by some other route than through vitamin B₁₂ (Becker et al., '49; Hale et al., '50; Marston and Lee, '49). In view of recent work (Koch and Smith, '51; Marston, '52; Smith et al., '51) it is apparent that administration of sufficient amounts of vitamin B₁₂ will produce a definite response in the cobalt-deficient sheep.

The purpose of this paper is to present the results of intravenous administration of vitamin B₁₂ to cobalt-deficient sheep, showing conclusively that vitamin B₁₂ alleviates the cobalt deficiency syndrome.

EXPERIMENTAL

In December of 1950 a group of spring lambs, after parasite treatment, were fed the cobalt-deficient ration described previously (Hoekstra et al., '52). The components of the ration were mixed together and full-fed the experimental animals. Six lambs were given supplemental cobalt by mixing 1 ounce of CoSO₄ · 7H₂O in each 100 lb. of salt. Records of weight gains, blood hemoglobin concentrations, and feed consumption were kept. When two lambs on the cobalt-deficient ration had lost about 25% of their body weight and had a blood hemoglobin concentration around 7 gm/100 ml or lower, they were isolated and fed individually. Following a short stabilization period, one of the animals was given 20 µg of vitamin B₁₂ twice daily for 21 days by intravenous administration (total dose of 840 µg). The quantity of cobalt in this amount of vitamin B₁₂ is far below that needed to get recovery by cobalt injection. An isotonic solution prepared from a 0.1% triturate of crystalline vitamin B₁₂ in NaCl² was used. The solution was sterilized by heating in boiling water for 5 minutes and was subsequently stored under refrigeration during the 21-day period of treatment. Following the first

² Kindly supplied by Merck and Co., Inc., Rahway, New Jersey.

treatment period, the untreated animal was made a member of another pair of experimental sheep treated in an identical manner. A total of three pairs of animals was used. Feed consumption, body weight and hemoglobin values were closely followed. Vitamin B₁₂ assays were made on the blood of two sheep before treatment and at intervals during the treatment period to determine if the vitamin B₁₂ injections were increasing the blood vitamin B₁₂ concentrations to "near normal" values. The blood samples were taken immediately preceding the first injection of a given day, 15 hours following the previous injection.

The treated animals were continued on the cobalt-deficient ration after vitamin B₁₂ injections were discontinued. They were then allowed to develop a second cobalt deficiency, whereupon they were again given intravenous injections of vitamin B₁₂, this time in doses of 40 µg once a day for 21 days. One of the sheep died of pneumonia before the second series of injections was initiated. During the depletion period one of the animals was given 3 mg of folic acid per day intramuscularly for a period of 16 days to determine if this factor had any hematological effect. The two sheep receiving the second series of vitamin B₁₂ injections were slaughtered 54 days after initiation of the second treatment. The blood, livers and rumen contents of these animals were analyzed for vitamin B₁₂ as described previously (Hoekstra et al., '52).

RESULTS AND DISCUSSION

Figures 1, 2 and 3 present the body weight, blood hemoglobin, and feed consumption data for the three sheep given vitamin B₁₂ injections. The first series of treatments were given in sequence, sheep No. 2 serving as a negative control during the injection period for sheep No. 1 and likewise, sheep No. 3 serving as a negative control for sheep No. 2. A negative control animal for sheep No. 3 died after losing 16 lb. during the period of 43 days following initiation of injections into experimental animal No. 3. From the figures it is evident that all three animals were in a severely cobalt-deficient

state at the time of the first treatment. Each had shown weight loss (about 20 lb.), depressed appetite, and a reduced blood hemoglobin concentration. In each case the intravenous injection of 20 μ g of vitamin B₁₂ twice a day brought about a

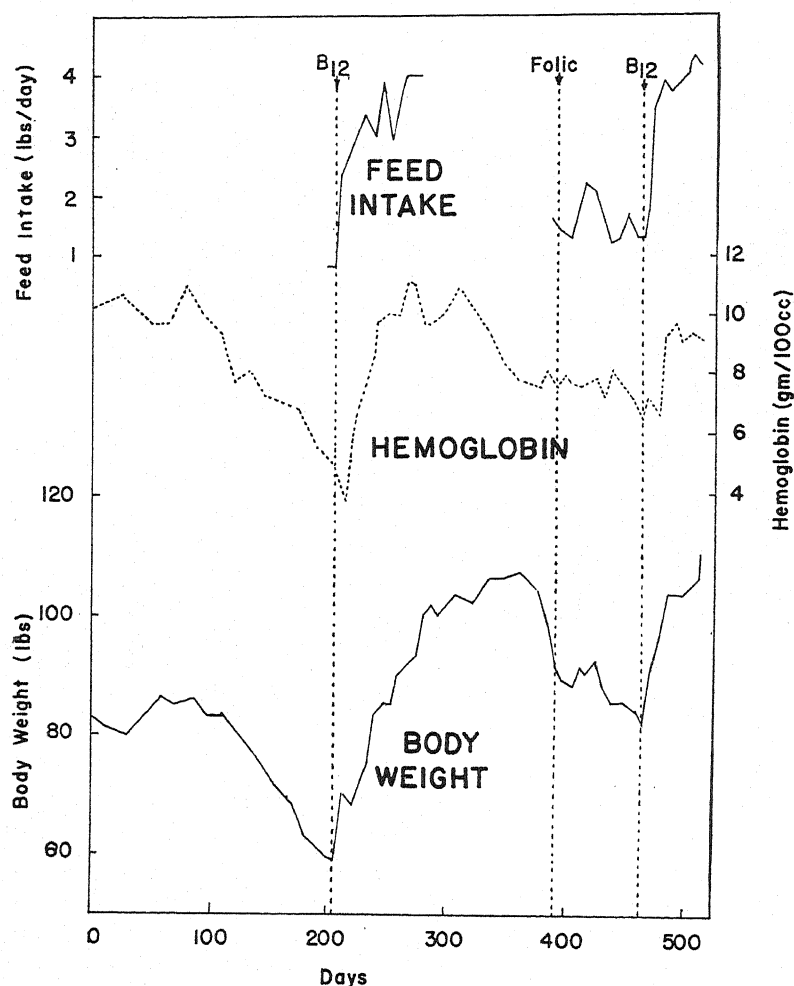


Fig. 1 Body weight, blood hemoglobin, and feed intake curves for sheep No. 1. The treatments consisted of intravenous injection of 20 μ g of vitamin B₁₂ twice per day for 21 days (initiated at 206 days); intramuscular injection of 3 mg of folic acid per day for 16 days (initiated at 390 days); and intravenous injection of 40 μ g of vitamin B₁₂ per day for 21 days (initiated at 463 days).

definite response. A remarkable increase in appetite (daily feed consumption tripled within one week) and a corresponding rapid and immediate weight gain were the first beneficial effects observed. During 80 days following initiation of treatments the three animals gained an average of 0.5 lb.

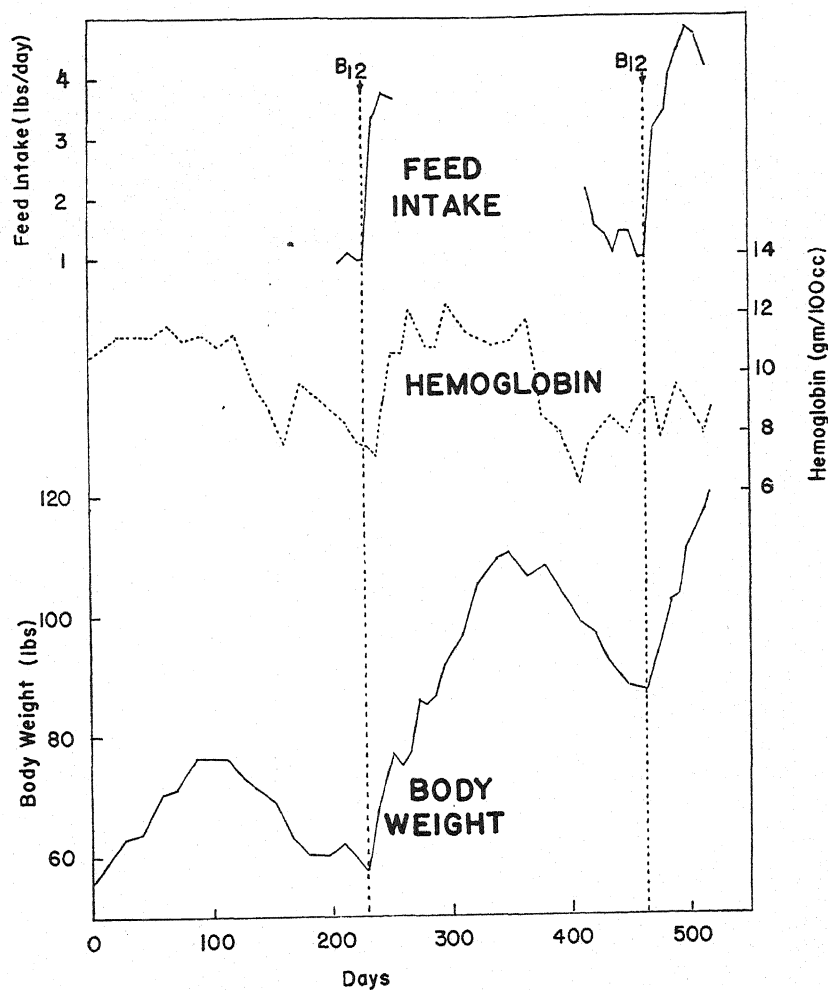


Fig. 2 Body weight, blood hemoglobin, and feed intake curves for sheep No. 2. The treatments consisted of intravenous injection of 20 μ g of vitamin B₁₂ twice per day for 21 days (initiated at 228 days) and 40 μ g of vitamin B₁₂ per day for 21 days (initiated at 463 days).

per day. The change from a dull, listless, and moribund animal to a bright-eyed, more vigorous animal was apparent within the first week. Hemoglobin responses were somewhat delayed, but in all instances returned to a normal value of 11 gm/100 ml or above within 40–60 days following initiation of vitamin B₁₂ injections. A very profound response occurred in sheep No. 1, whose hemoglobin concentration rose from

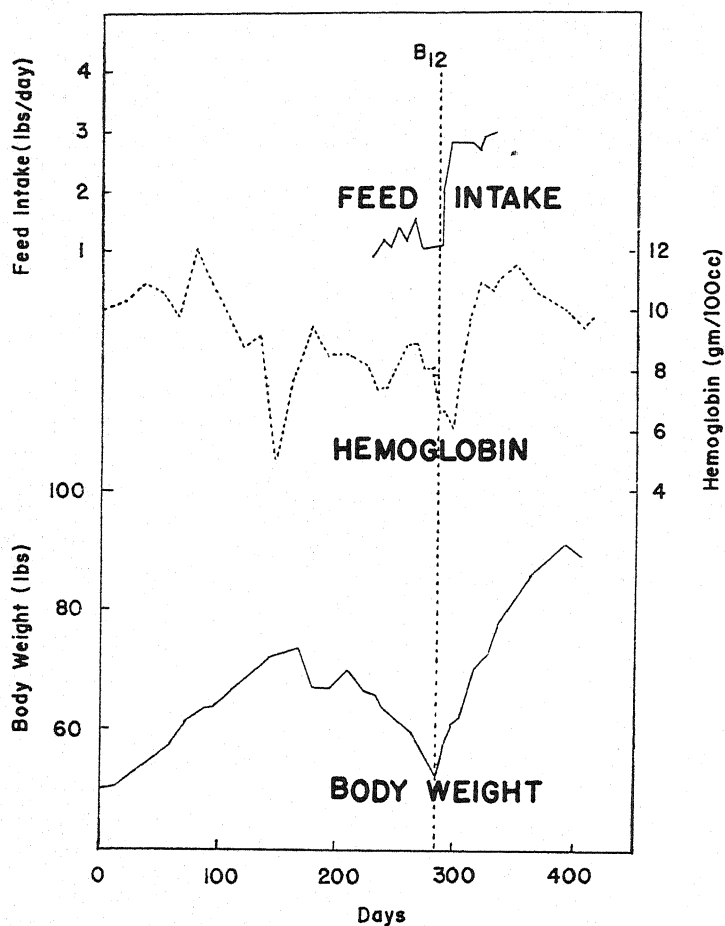


Fig. 3 Body weight, blood hemoglobin, and feed intake curves for sheep No. 3. The treatment consisted of intravenous injection of 20 μ g of vitamin B₁₂ twice per day for 21 days.

a low of less than 4 gm/100 ml to above 11 gm/100 ml following vitamin B₁₂ administration. These observations show that vitamin B₁₂ was capable of producing a definite hemoglobin response in the cobalt-deficient sheep. Numerous investigators have reported delayed and erratic hemoglobin responses after administration of cobalt to cobalt-deficient sheep (Becker and Smith, '51; Filmer, '33; Filmer and Underwood, '37; Ray et al., '48). Koch and Smith ('51) and Smith et al. ('51) have observed a similar erratic hemoglobin response following administration of vitamin B₁₂ to cobalt-deficient sheep. Sheep No. 2 did not respond significantly in blood hemoglobin concentration within 54 days following the second series of vitamin B₁₂ injections. The reason for these delayed and erratic hemoglobin responses in certain instances is not clear. It may indicate the lack of some other hemopoietic factor, or possibly complicating factors such as internal parasites. Spontaneous partial remissions in the hemoglobin curves as observed for sheep Nos. 2 and 3 at about 150 days are often observed in cobalt-deficient sheep when hemoglobin values are closely followed.

The amount of vitamin B₁₂ administered was based upon that amount estimated to be required to maintain normal blood concentrations. Vitamin B₁₂ analyses of blood samples from animals 1 and 2 before and at intervals during the injection period showed that the concentrations of vitamin B₁₂ in the blood of each animal rose from about 0.4 mug/ml to between 1.0 and 2.0 mug/ml of whole blood. In another publication (Hoekstra et al., '52) we have presented the average blood vitamin B₁₂ concentration of 16 sheep on the experimental ration early in cobalt deficiency as 0.47 mug/ml and that of 6 sheep full-fed the same ration plus supplemental cobalt as 2.3 mug/ml. Thus, this selected dosage of vitamin B₁₂ had raised the blood vitamin B₁₂ to "near normal" values. No deficient animals were fed cobalt for a direct comparison of a cobalt response with the intravenous vitamin B₁₂ response observed in this study. However, the 6 animals on the same ration given supplemental cobalt from the beginning of the

experiment had gained at 210 days an average of 54 lb. (0.26 lb./day). Their hemoglobin levels were normal. This demonstrated that cobalt was definitely the limiting factor in this ration. The responses attained with vitamin B₁₂ in this experiment are as good as or better than responses reported for cobalt supplementation to deficient sheep (Becker and Smith, '51; Smith et al., '51; Ray et al., '48; and many others). Koch and Smith ('51) have demonstrated equivalent responses of cobalt-deficient sheep to injected vitamin B₁₂ and orally administered cobalt.

The beneficial effects of the vitamin B₁₂ administrations on body weight gains continued for about 100 days following initiation of the treatment. At this time the weight curves leveled off, and soon the animals were losing weight and blood hemoglobin concentrations were falling. Animal No. 3 died of pneumonia at this time.

The administration of folic acid to sheep No. 1 is more or less incidental to this experiment. The well-known response to folic acid in pernicious anemia in the human, which is apparently a result of failure to absorb vitamin B₁₂, suggested the possibility that folic acid given in large amounts might have some effect on the hemopoietic status of the cobalt-deficient sheep. Sheep No. 2 showed little or no response to the folic acid. However, conclusions should not be made on the basis of this one animal. Studies on the relationships of other hemopoietic factors including folic acid to vitamin B₁₂ in the cobalt-deficient sheep are in progress.

The second series of vitamin B₁₂ injections to sheep Nos. 2 and 3 after developing a second cobalt deficiency again produced dramatic responses in weight gains and feed consumption. The hemoglobin response was suboptimal at 54 days after initiation of the treatment in sheep No. 1, and was absent in sheep No. 2. These two animals were slaughtered at this time. The results of assays for vitamin B₁₂ in the blood, livers, and rumen contents of these animals are shown in table 1. The vitamin B₁₂ values for cobalt-deficient sheep on the experimental ration, reported in another publication

(Hoekstra et al., '52), were approximately 0.5 mug vitamin B₁₂ activity/ml whole blood, 0.03 μ g "true" vitamin B₁₂ activity/gm moist liver (total activity minus alkali stable activity), and 0.09 μ g vitamin B₁₂ activity/gm dry rumen contents. The vitamin B₁₂ injected sheep showed slightly higher vitamin B₁₂ concentrations in the blood, about three times as much "true" vitamin B₁₂ activity in the liver, but the same amount in the rumen ingesta. These two animals were gaining weight and consuming normal amounts of feed at the time of slaughter. If the total response to the second vitamin B₁₂ treatment is of the same magnitude as the response to the first treatment, and if the greater body weights of the animals at this time are considered, the animals could

TABLE 1

Vitamin B₁₂ concentrations in the blood, livers and rumen contents of sheep nos. 1 and 2 at the time of slaughter

SHEEP NO.	VITAMIN B ₁₂ ACTIVITY OF WHOLE BLOOD	TOTAL VITAMIN B ₁₂ ACTIVITY OF MOIST LIVER	ALKALI STABLE VITAMIN B ₁₂ ACTIVITY OF MOIST LIVER	VITAMIN B ₁₂ ACTIVITY OF RUMEN CONTENTS
	$m\mu g/ml$	$\mu g/gm$	$\mu g/gm$	$\mu g/gm$ dry wt.
1	0.64	0.106	0.007	0.08
2	0.61	0.073	0.007	0.10

not have been far from the limit of response to the second vitamin B₁₂ treatment. This is substantiated by the blood and liver analyses. The liver vitamin B₁₂ values also suggest that the duration of response to vitamin B₁₂ treatment probably results from a storage of this factor during the treatment period.

This experiment represents a period in excess of 500 days during which sheep consumed a cobalt-deficient ration. The remarkable responses of the deficient animals to the initial vitamin B₁₂ treatment and the similar response in a second deficiency suggests that vitamin B₁₂ is by far the most important, if not the only, limiting factor in a cobalt deficiency in sheep. The erratic hemoglobin responses found after the second vitamin B₁₂ treatment and those reported by Cornell workers

(Koch and Smith, '51; Smith et al., '51) to vitamin B₁₂ treatment do not discredit this conclusion, since similar responses have been widely reported on administration of cobalt to cobalt-deficient sheep.

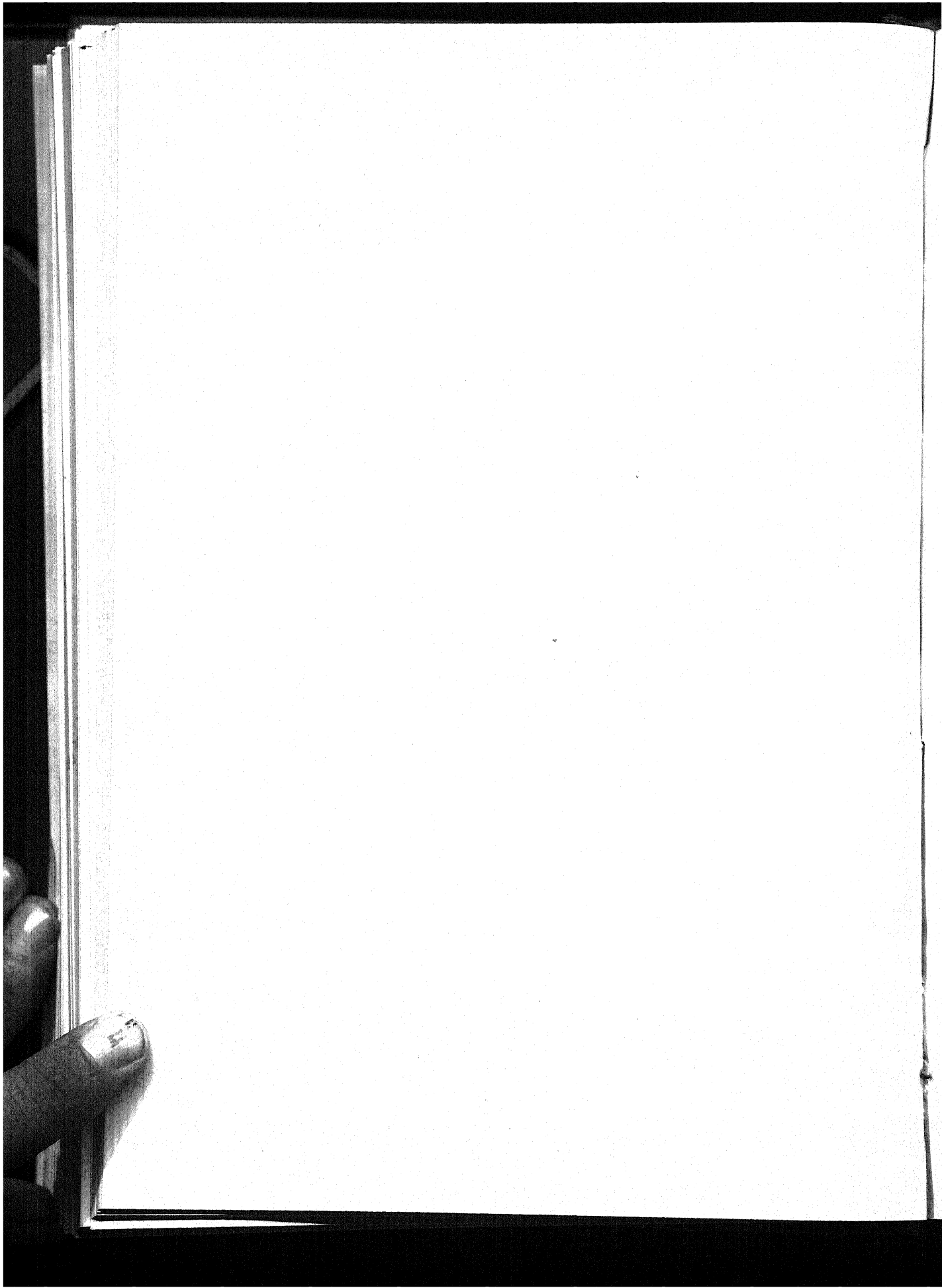
SUMMARY

Three sheep in a severe cobalt-deficiency as characterized by anemia, body weight loss, lack of appetite, and general moribund condition, were given intravenous injections of 20 µg of vitamin B₁₂ twice per day for 21 days. In all of the cases this treatment was followed by an immediate and remarkable response in feed consumption, vigor, and body weight gain. Blood hemoglobin concentrations rose to normal within 40 to 60 days. The response to treatment continued for about 100 days; then the animals began developing evidence of a second cobalt-deficiency. Two of the sheep were given a second series of vitamin B₁₂ injections (40 µg/day for 21 days), and again exhibited similar responses although the hemoglobin responses were somewhat erratic. The fact that these injections of vitamin B₁₂ brought about such profound responses to sheep fed a cobalt-deficient ration for more than 500 days suggests that vitamin B₁₂ is by far the most important, if not the only, limiting factor in the cobalt-deficient sheep.

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THE UTILIZATION OF CALCIUM FROM LACTATE, GLUCONATE, SULFATE AND CARBONATE SALTS BY YOUNG COLLEGE WOMEN ^{1, 2}

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ONE FIGURE

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Calcium salts are often used as a source of calcium for comparison in experiments designed to study utilization of calcium from different foods. It is also a common practice for the physician to recommend the use of tablets of calcium salts to supplement that from food sources at times when large amounts are needed, such as during pregnancy and lactation. It has been found in studies of the dietary habits of older persons that many of them consume diets inadequate in calcium, and if they have not been accustomed to drinking milk, it is difficult to get them to increase their calcium intake by this means. Calcium salts have been used in such cases. In order to improve diets of the general population, particularly under emergencies such as war conditions, calcium salts have been used in the enrichment of breadstuffs and meat products. Under still other conditions, due to unequal distribution or inadequacies of food supplies, calcium salts have been recommended as a means of increasing calcium intakes. Hence it seemed worthwhile to compare the utilization of calcium from some of the salts that are used as supplements.

¹ This paper is condensed from the dissertation submitted by Mary Brown Patton in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Foods and Nutrition in the Graduate School of The Ohio State University.

² Journal Article 35-52, Ohio Agricultural Experiment Station.

Studies in which salts have been the sole source of calcium in the diet have been limited almost entirely to those with experimental animals since it is very difficult to obtain permission of human beings to subsist on a purified diet for a sufficient length of time to obtain reliable results. However, in one study three human subjects were maintained for 7 weeks on a purified diet in which the calcium was furnished by a mixture of phosphate and carbonate salts at a level of 879 mg of calcium daily. A natural diet supplied 789 mg daily for a three-week period (Hathaway, '49). The calcium from food sources was better utilized than that from the salts in all three cases.

Studies have been made with human beings in which the calcium in a diet of natural foods has been supplemented by that from various salts. The purpose of most of these studies has been to compare the utilization of calcium from food sources rather than that from the different salts. Two studies made among young children may be cited in which the generalization was reached that calcium from salts is retained as well as that from milk (Kempster et al., '40; Stearns and Jeans, '34). Several studies made among adults may be cited in which it was shown that calcium in salts was utilized to approximately the same extent as that in milk (Drake et al., '49; McQuarrie et al., '47; Schroeder et al., '46; Steggerda and Mitchell, '39, '46). In another study (McCance and Widdowson, '42) the conclusion was reached that the form of the salt used in bread as enrichment was not important, but that the type of flour was a factor due to the amount of phytic acid present.

PROCEDURE

The young women selected as subjects had been college students for at least one year, had no strong dislikes or allergies for foods in the diet, and all were considered healthy by the examining physician.³

³ Dr. Keith Frankhauser, medical officer in charge of a Nutrition Unit of the U. S. Public Health Service stationed in Ohio in 1948-1950.

The diet differed from day to day but remained the same from week to week. Each individual food was weighed for each subject although certain ones may have been combined and served. Aliquots of a weekly food composite were dried in an oven at 60°C. and samples were burned in an oxy-calorimeter. The diet contained an average of 2179 cal. daily according to this method of determination (Fuqua, '48). Those subjects who required additional calories were given weighed amounts of foods of low calcium content. The fact that all of these young women maintained their weight within the expected fluctuations of slight weekly gains and losses indicates that the diet was probably adequate in calories.

The protein content of the diet was determined by the method of the Association of Official Agricultural Chemists ('45); the calcium by the Stearns ('29) modification of the McCrudden method; and the phosphorus by the Fiske and Subbarow ('25) molybdate method. The diet supplied an average of 57 gm protein, 347 mg calcium, and 758 mg phosphorus daily.

The ascorbic acid, thiamine, and riboflavin contents of the diet were calculated from food tables. The diet was estimated to be adequate in all respects except riboflavin and calcium. To bring the riboflavin intake to the recommended level each subject received one milligram of riboflavin daily in tablet form. The basal diet, containing 347 mg of calcium per day, was supplemented by 400 mg of calcium daily from the salts which were added in approximately equal portions at the beginning of each meal. The total of approximately 700 mg was the amount considered to be just below the critical level, that level above which an additional dietary increment would not be expected to result in additional storage but have a retention value of zero. As recommended by the Food and Nutrition Board of the National Research Council ('48), the diet was further supplemented with 500 I.U. of vitamin D in the form of "Viosterol" given daily at breakfast time.

Excreta were collected according to the customary procedures. Carmine was used as a fecal marker. Calcium was

precipitated from aliquots of an acidified composite of urine. Aliquots of feces and food composites were wet ashed and calcium determined by a modification of the McCrudden method as described by Stearns ('29).

TABLE 1
Plan of study of the utilization of 4 calcium salts

PERIOD	LENGTH OF PERIOD	SUBJECTS				
	<i>day</i>					
I	2-7	{ ^I No sup- plement	^{II} No sup- plement	^{III} No sup- plement	^{IV} No sup- plement	^V No sup- plement
I	2-7	{ ^{VI} No sup- plement	^{VII} No sup- plement	^{VIII} No sup- plement	^{IX} No sup- plement	
II	2-7	{ ^I Lactate	^{II} Sulfate	^{III} Gluconate	^{IV} Carbonate	^V Gluconate
II	2-7	{ ^{VI} Sulfate	^{VII} Lactate	^{VIII} Carbonate	^{IX} Gluconate	
III	2-7	{ ^I Carbonate	^{II} Lactate	^{III} Sulfate	^{IV} Gluconate	^V Carbonate
III	2-7	{ ^{VI} Gluconate	^{VII} Sulfate	^{VIII} Lactate	^{IX} Carbonate	
IV	2-7	{ ^I Gluconate	^{II} Carbonate	^{III} Lactate	^{IV} Sulfate	^V Lactate
IV	2-7	{ ^{VI} Carbonate	^{VII} Gluconate	^{VIII} Sulfate	^{IX} Lactate	

Serum-calcium levels were followed for each subject on blood samples collected at the beginning of the study and at the close of each experimental period. Determinations were made in the laboratory of the Ohio Department of Health.⁴

The Statistical Laboratory of The Ohio State University planned an experimental design (table 1) in which 8 subjects (Subjects I to VIII, inclusive) were to be studied. A 9th subject was included and she received the supplements indicated in table 1.

⁴ These determinations were made by E. C. Tabor, chief chemist with a Nutrition Unit of the U. S. Public Health Service stationed in Ohio in 1948-1950.

RESULTS AND DISCUSSION

The calcium balances of 9 subjects during periods II, III, and IV are reported in table 2. A statistical analysis of the data for Subjects I to VIII, inclusive, was made by the method of least squares and no significant effects of salt, individual, or time (order in which the various salts were taken) could be found. If it is assumed that significant differences do exist, the question arises as to why these data fail to reveal such differences. These suggestions are offered: too few subjects, sensitivity of statistical method, and individual differences offset treatment differences.

TABLE 2

Calcium balances of 9 young women during three experimental periods when a low calcium diet was supplemented by 400 mg of calcium from salts

PERIOD	SUBJECT								
	I	II	III	IV	V	VI	VII	VIII	IX
	mg	mg	mg	mg	mg	mg	mg	mg	mg
II	24	70	76	47	17	32	-25	53	73
III	-90	-13	65	18	-48	10	6	88	-1
IV	-4	-48	-61	-27	-83	-36	157	18	-60

Among the differences in expected and observed values of the 24 balances of these 8 subjects, the two largest were associated with Subject VII. Observation of the values in table 2 reveals that this subject did show unusual variation, particularly as far as the time element was concerned. Inspection of the fundamental data shows that she was in negative balance during the first experimental period while all the other subjects were in positive balance. During the third experimental period this subject showed a comparatively large positive balance and all other subjects, except one, were in negative balance.

If Subject VII is replaced by Subject IX, who was observed at the same time, the symmetry in treatments will be lost. However, if it is assumed that the treatment effects are zero, an analysis of these new data may be made to see if the

statistical method used would then reveal any significance in time or in individual subject differences. When this replacement was made the mean retention on the sulfate treatment was increased from 27.33 to 31.60 mg; the mean for the lactate treatment was reduced from -11.67 to -17.50 mg; the mean for carbonate treatment was increased from -20.30 to -17.57 mg; and the mean for the gluconate treatment was decreased from 45.67 to 31.67 mg.

Based on the values when Subject IX is substituted for Subject VII, it was shown by analysis that the individual differences in the subjects cannot be considered as significant but that time produced a difference in calcium balances among these subjects that is significant at the 5% level.

Further analysis of the data, using all 9 subjects, was made to determine the percentage of utilization of each salt according to the method used by Steggerda and Mitchell ('46). In 4 cases the balance on a supplement was less than that on the basal diet alone and these cases were termed zero utilization. The mean value for carbonate utilization was 10.79%; for sulfate, 20.92; for lactate, 11.45; and for gluconate, 28.79. The less soluble salts, carbonate and sulfate, were as well utilized as the more soluble ones, gluconate and lactate. Schroeder et al. ('46) reported an average of 23.65% utilization of the calcium of calcium sulfate. Average utilizations of 20 and 25% have been reported for gluconate in two studies by Steggerda and Mitchell ('39, '46). Stearns and Jeans ('34), in a study with young children, reported variable results for lactate, gluconate and carbonate but fairly consistent results with phosphates. They reasoned that the difference was due to the variation brought about in the calcium-phosphorus ratio. The senior author of the present study was unable to relate balance to calcium-phosphorus ratio in a study among a group of 18 young college women (Patton et al., '52). The values reported above for the 4 salts used in the present study are within the range reported by these other workers and within the range of that reported for milk, the food recognized by nutritionists as the best source of calcium.

In studying the percentage utilization of the salts by the 9 subjects reported herein, it was noted that 7 of the 9 subjects utilized to the largest percentage the salt taken first, regardless of which salt it was (fig. 1). The correlation coefficient of utilization and rank was 0.67 which is significant for this number of cases. The time at which a salt was taken was shown to be a factor when the data for the 8 subjects (Subject IX substituting for Subject VII) were analyzed by the method of least squares.

Blood samples were taken from each subject at the beginning of the study and every two weeks thereafter and analyzed for

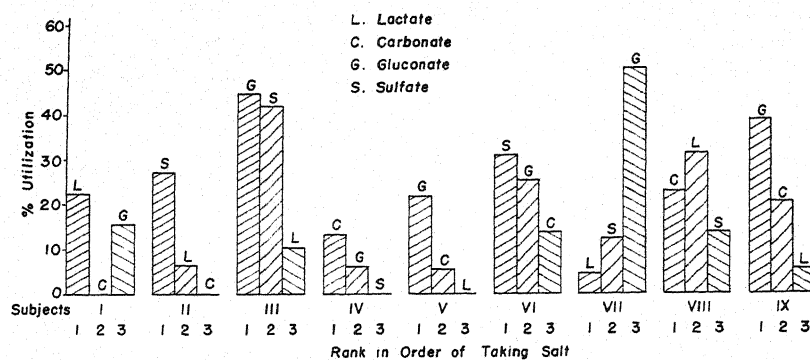


Fig. 1 Relation of the per cent utilization to the rank in order of taking a salt.

serum-calcium. The average value at the beginning was 9.8 mg % and at the end of the no-supplement period, 10.5. The levels for the periods when each of the salts was given are practically the same, 11.3, 11.5, 11.5, and 11.8, respectively, for gluconate, carbonate, lactate, and sulfate. All of the values were within the normal range of 9 to 11 mg %. The increase in blood level that was noted when 400 mg of calcium were added to their diets as salts is not in agreement with the statement by Sherman ('47) that "in normally nourished individuals, the intake of food calcium or even the administration of calcium salts alone has hardly a measurable direct influence upon the blood calcium level."

The average utilization of calcium from the three salts taken by each subject was compared with the basal heat production per square meter per 24 hours. Three subjects, whose basal metabolic rate was slightly above the Aub-DuBois predicted standard but within normal range, showed an average utilization of 22.7%. A 4th subject, whose rate was slightly below the standard but within the normal range, showed an average utilization of 21.5%. The basal metabolic rates of the remaining 5 subjects were below their predicted standard by from 7 to 27%. The average utilization of the salts by these 5 subjects was only 14.0%. These results suggest a possible relationship between calcium utilization and basal metabolic rate. Johnston and Maroney ('36) have shown that poor diets in general result in low energy metabolism. Although a record was made of the foods eaten by each subject in the study reported herein on the day previous to the beginning of the study, it has not been considered as an evaluation of their customary eating habits. This observation emphasizes the importance of noting previous dietary habits in studies such as the one reported.

The difficulty of separating feces into portions that represent those from the several periods offers a possible source of errors in studies such as this one. Two subjects, who were very regular in their habits of elimination, had formed stools in which the carmine usually appeared in from 24 to 36 hours, and the demarcation was clear cut, showed a fairly consistent utilization of calcium from the salts that supplemented their diets. One subject was constipated and the carmine usually remained in the digestive tract for from 4 to 5 days. However, this subject showed the greatest average percentage retention of calcium of any of the subjects studied. The remaining 6 subjects varied considerably as to the nature of their stools and the time of passing the carmine.

Leichsenring et al. ('51) reported that a group of 18 college women were able to adjust to the level of calcium in a basal diet similar to the one used in this study within one week. For this reason that period was used for each of the experi-

mental periods in this study. The data show that, regardless of the nature of the salt taken as a supplement, if it happened to be the first salt following the preliminary adjustment period when the intake was 300 mg, its retention was greater than that for the same or any other salt at a later period. This casts some doubt as to whether or not these 9 subjects made the adjustment from the 300 to the 700 mg level during the one week allowed for adjustment.

Another implication from these data might be that human beings do not efficiently utilize calcium from salts for periods of more than a couple of weeks. A study has been carried out in this laboratory to obtain additional information on this subject and a report of the findings is in preparation.

SUMMARY

A study was made of the utilization of calcium by 9 college women from 4 salts (gluconate, lactate, carbonate, and sulfate) given in amounts equivalent to 400 mg of calcium daily. The basal diet contained an average of 347 mg of calcium daily and calculation of other nutrients showed that the diet was adequate.

Statistical analysis of the balance data was made by the method of least squares. No significant differences in the utilization of calcium from these salts were found, the calcium from the less soluble salts being utilized as well as that from the more soluble ones.

The order in which the salts were taken was shown to be a significant factor in the utilization. In the majority of the cases, the salt taken first was utilized to the largest percentage regardless of which salt it was.

The average percentage utilization from all salts for all subjects was approximately 18%, a value within the range of those reported in the literature for utilization of calcium from food sources.

Young women having normal basal metabolic rates showed somewhat greater percentage utilization of calcium from salts than those whose rate was below the predicted normal.

The serum-calcium levels were normal, 9 to 11 mg per 100 ml, during the 8 weeks of the study and were practically the same for the periods when the 4 salts supplemented the basal diet.

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EFFECT OF UNTREATED CORN AND MEXICAN
TORTILLA UPON THE GROWTH OF RATS
ON A NIACIN-TRYPTOPHAN
DEFICIENT DIET

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Krehl et al. ('45a, b) observed that corn has a growth-depressing effect upon the rat when added to a niacin-tryptophan low diet. It has been questioned (Krehl, '49) whether that action is due to a toxic factor present in corn or is conditioned by an amino acid imbalance. Several authors (Henderson et al., '47; Krehl et al., '46a, b; Rosen and Perlzweig, '49; Singal et al., '48) have demonstrated similar effects with either amino acid mixtures, tryptophan deficient proteins or acid hydrolysates of proteins. Recently, Hankes et al. ('49), and Lyman and Elvehjem ('51) showed that mixtures of gelatin and some amino acids have the same growth-depressing action when they are fed in the presence of either L-cystine or methionine. Corn is used in México as human food largely in the form of tortillas and one of the steps in the preparation of this food is by heating it for one hour at 80°C. with lime water which is discarded after a 24-hour period; only the softened grain being ground and used (Cravioto et al., '45).

Krehl et al. ('46a) have reported that tortilla has approximately the same growth-depressing effect as untreated corn, but Laguna and Carpenter ('51) have recently reported that corn heated with lime water loses its depressing effect when

incorporated into rations similar to those used by Krehl and his co-workers. They attributed this loss to the liberation of nicotinic acid from a "precursor" when the grain was heated in lime water, *without, however*, presenting any supporting data. Furthermore, the preparation used by Laguna and Carpenter was not the same as the Mexican tortilla, since the heated grain and mother liquors were dried together and fed as such after neutralization with phosphoric acid. In the present study a comparison was made between the effect of untreated corn and Mexican tortilla upon the growth of rats on a niacin-tryptophan deficient diet, with and without supplements of cystine.

EXPERIMENTAL

A basal diet similar to that used by Krehl et al. ('45b) was prepared, but the level of casein was 11.66%, and the salt mixture of Hubbell et al. ('37) was utilized instead of the Phillips and Hart mixture. Vitamins were incorporated into the diet at the same levels used by Krehl and his co-workers. Casein (Baker's) was extracted 5 times with hot 96% ethyl alcohol and the content of nicotinic acid after this treatment was found to be less than 20 μ g per 100 gm.

Six rations were prepared from the basal diet by mixing 60 parts with 40 parts of the following supplements: corn starch (ration Ia); white corn (ration IIa); air-dried tortilla, prepared according to the Mexican fashion as described by Cravioto et al. ('45) (ration IIIa); rations Ib, IIb, and IIIb were the same except for the addition of 10 mg of nicotinic acid per kilogram.

Five other rations consisted of 60 parts of the basal diet with L-cystine omitted, and 40 parts of: corn starch (ration IVa); white corn (ration Va) or air-dried tortilla (ration VIa). Ten milligrams of nicotinic acid were added per kilogram of rations IVa and Va to obtain rations IVb and Vb. The final level of casein was 7% in all rations.

Sixty-six weanling albino rats, Wistar strain, weighing 35 to 40 gm were distributed in 11 groups of 6 animals (three

males and three females) each placed individually in galvanized iron cages with wire bottoms, and each group was fed one of the rations. Food and water were given ad libitum, and the food consumption was recorded. Two drops of a corn oil solution of vitamin A (4,200 I.U./ml), vitamin D (420 I.U./ml), and α -tocopherol (0.7 mg/ml) were given to each rat weekly. During the 28-day experimental period the animals were weighed three times a week.

Nicotinic acid was determined by the U.S.P. microbiological method ('43) in the corn and tortilla as well as in a corn preparation similar to that described by Laguna and Carpenter ('51).

The results were expressed as final weights as weight gain per 100 gm of food consumed.

RESULTS AND DISCUSSION

The weight gained per 100 gm of ration Ia, which contained starch, was used as the basis for evaluating the effect of the other rations. The replacement of starch by corn resulted in a similar growth in spite of the higher level of protein in the corn ration (table 1, groups Ia and IIa). However, when starch was replaced by dried tortilla, growth was much higher than that of the animals on the corn ration (table 1, groups IIa and IIIa). Statistical analysis of the data shows that this difference is significant. The ratio of the mean difference in growth to the probable error of the difference is 13.5. The addition of 1 mg % of nicotinic acid to the corn ration produced growth equal to that obtained with the tortilla ration, but niacin did not increase growth on this latter ration.

As was expected, lower growth was obtained with ration IVa (basal + starch) from which L-cystine was omitted. It is particularly noticeable that with the omission of cystine the corn and tortilla rations produced more nearly equal rates of growth, the growth-depressing effect of corn being apparently lost. The difference in growth between groups Va and VIa is not statistically significant. It should be noted

TABLE 1
Growth of rats on the different rations studied

RATION	SUPPLEMENT	ADDED NIACIN	INITIAL WEIGHT ¹ gm	FINAL WEIGHT ¹ gm	FOOD CONSUMED DAILY PER RAT ¹ gm	WEIGHT GAINED PER 100 GM OF FOOD CONSUMED ¹ gm
Ia	Corn starch	..	37.9 ± 0.7	73.1 ± 2.1	7.5 ± 0.3	16.7 ± 0.7
IIa	Corn	..	36.6 ± 0.5	67.3 ± 2.5	6.0 ± 0.1	18.0 ± 0.4
IIIa	Tortilla	..	37.8 ± 0.9	107.3 ± 3.1	9.3 ± 0.3	26.5 ± 0.5
Ib	Corn starch	1	36.8 ± 0.5	63.2 ± 4.6	6.9 ± 0.5	13.5 ± 1.5
IIb	Corn	1	38.1 ± 0.9	116.1 ± 1.9	10.2 ± 0.4	26.2 ± 0.2
IIIb	Tortilla	1	37.4 ± 0.8	108.2 ± 3.5	9.3 ± 0.4	27.2 ± 0.9
IVa	Corn starch n/c ²	..	36.8 ± 0.5	59.4 ± 1.8	6.3 ± 0.5	12.7 ± 0.6
Va	Corn n/c	..	36.4 ± 0.9	77.5 ± 4.4	6.3 ± 0.4	23.1 ± 1.1
VIa	Tortilla n/c	..	35.7 ± 0.6	95.6 ± 3.6	8.4 ± 0.4	25.5 ± 0.8
IVb	Corn starch n/c	1	36.5 ± 1.1	48.8 ± 1.5	5.5 ± 0.3	8.1 ± 0.6
Vb	Corn n/c	1	36.6 ± 0.9	98.0 ± 2.9	9.3 ± 0.1	23.6 ± 0.4

¹ Including the probable error of the mean result calculated according to the formula $\sqrt{\sum d^2/n} - 1/\sqrt{n} \times 0.6745$, where "d" is the deviation from the mean and "n" is the number of observations (Sherman, '41).

² n/c means cystine omitted (see the text).

that there is a lower gain in weight, and less food intake, in the animals fed the corn ration with cystine omitted. Therefore at least two variables may be involved in these results: one a function of food efficiency, another a function of food intake. This makes it difficult to interpret the data obtained. Nevertheless, if the groups fed diets IIa (with cystine) and Va (without cystine) are compared, it can be seen that the animals fed ration Va had a greater gain in weight than those in group IIa. It should be noted that in this case there was practically no difference in the food intake. Statistically, the difference between weight gained in groups IIa and Va is significant. The ratio of the mean difference in growth to the probable error of the difference is 4.3. It is possible that the growth-depressing effect of corn is due to an amino acid imbalance shown in the presence of cystine. This would be in agreement with the observations of Hankes et al. ('49), and Lyman and Elvehjem ('51).

The variations in food intake of the different rations make it difficult to interpret the results. It should be noted that in most of the reports by Krehl et al. ('45a,b, '46a, b) and also in the report of Laguna and Carpenter ('51) the food intake by the animals is not given. This makes it hard to compare their results with ours. The possibility exists that the differences in weight gain of the animals fed the different rations were exaggerated by the variations in food intake. Some of the groups did not eat very well, possibly because of unpalatability of the ration due to physical texture or taste or other unknown causes. Therefore, in certain cases some degree of starvation can operate as a determining factor in protein utilization. It has been reported (Barnes et al., '45) that a restriction of the protein intake from diets low in these substances causes a decrease in their utilization.

The nicotinic acid contents of the corn and tortilla were found to be 2.33 and 2.15 mg %, respectively, as measured by microbiological assay. Liberation of more of this vitamin

could not be noted after treatment of the corn by the Laguna and Carpenter ('51) procedure.

Since no liberation of niacin was observed by treatment of the corn with hot lime water, the lack of a growth-retarding effect in tortilla cannot be explained on the basis of the Laguna and Carpenter ('51) hypothesis. It may be due to destruction or racemization of certain amino acids in the corn during the treatment with lime water. These changes appear to be most marked for threonine, arginine, and histidine (Massieu et al., '49), and they might be enough to minimize the amino acid imbalance of untreated corn. Nevertheless, because of the low intake of the corn ration, some degree of starvation may have operated as mentioned above, with less protein being made available for growth, and therefore, a lower figure being obtained for the last column. Hence this lower figure is not necessarily due to amino acid imbalance. Further study of the problem is needed in order to settle the question.

The reason for the disagreement between the growth-depressing effect of tortilla reported by Krehl et al. ('46a) and the results of Laguna and Carpenter ('51) as well as those presented in this paper might be due to differences in the treatment of the corn; but data would be necessary to find the actual reason for such a discrepancy.

SUMMARY

A study was made of the effect of corn and Mexican tortilla upon the growth of weanling rats fed diets low in niacin and tryptophan, with and without supplements of cystine.

Corn inhibited growth in cystine-supplemented rations, but tortilla actually stimulated growth. In the absence of cystine corn apparently failed to depress the growth, which was similar to that in rats fed tortilla.

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THE RELATIONSHIP BETWEEN THE PROTEIN CONTENT OF CORN AND THE NUTRITIONAL VALUE OF THE PROTEIN

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The protein content of corn on the dry basis may be varied widely by selective breeding, by weather conditions, and by crop and soil management practices. At the Illinois Agricultural Experiment Station, starting with a variety of corn containing 10.92% protein, selection through 50 generations for high- and low-protein characteristics has produced a high-protein strain averaging 19.45% protein, and a low-protein strain averaging 4.91% (Woodworth, Leng and Jugenheimer, '52).

The effect of crop yield, soil type and treatment and seasonal conditions on the composition of corn and other grains has been studied by Stubblefield and DeTurk ('40). In an intensive investigation of the effects of soil treatment and crop rotation on the composition of corn, Hamilton, Hamilton, Johnson and Mitchell ('51) observed a 40% increase in the protein content of the same varieties of corn during the same season under the best conditions of soil and crop management over that under the worst conditions imposed (10.4% as compared with 7.5% on the dry basis).

Hopkins, Smith and East ('03) were the first to demonstrate that changes in the protein content of corn induced by selective breeding are related to changes in the proportions existing among the anatomical parts of the kernel.

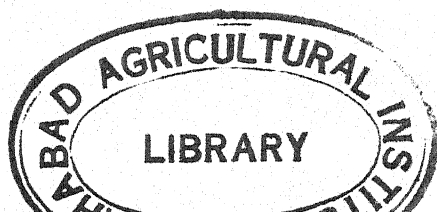
¹ Deceased.

The most prominent change in the physical composition accompanying increasing protein content is a greatly increased proportion of the horny over the starchy endosperm. This anatomical change was observed by Hamilton et al. ('51) to accompany increased protein content of corn induced by changes in soil treatment and crop management. It was also shown in this investigation (1) that the horny endosperm contains a higher concentration of protein than the floury endosperm, (2) that all the major parts of the kernel contribute, though in different degrees, to the increase in the total protein of the corn kernel, and (3) that the alcohol-soluble protein, zein, is for all practical purposes confined to the endosperm, the horny endosperm holding 60 to 65% of the endosperm zein. One would therefore expect the zein content of corn to increase in percentage more rapidly than does the total protein under conditions favoring the production of high-protein corn.

The first to discover the relationship between the proportion of zein in corn protein and the nitrogen content of the whole kernel were Showalter and Carr ('22). Hansen, Brimhall and Sprague ('46) defined this relationship more precisely for 18 different corn samples covering the range 6.3 to 19.7% protein. They established a correlation coefficient of +0.92 and a linear regression of zein content on total protein content. Frey ('51), working in the same laboratory with 90 to 100 samples of corn ranging in protein content from 4.9 to 18.4%, also observed a high correlation between the percentage of zein and the percentage of total protein. However, this author chose to describe the relation of these two variables with a simple parabolic equation of the type used by Huxley ('32) to describe the phenomenon of relative growth, i.e., $y = ax^b$, without showing that the parabolic equation gave a better fit than would a linear equation. However, in the chosen equation, the value of b , 1.87, was significantly greater than 1, testifying to the more rapid increase of zein than of total protein in the corn kernel.

The demonstration that the proportion of zein in the total protein of corn is directly correlated with the content of protein in corn logically led to studies of the amino acids in corn because of the nutritionally unbalanced character of the essential amino acids in zein. The problem was studied from the genetics standpoint by Doty et al. ('46), with results indicating that "... it might be possible by careful breeding to develop a hybrid with a high proportion of nutritionally essential amino acids in its protein . . ." Miller, Aurand and Flach ('50) determined lysine, methionine and tryptophan contents of corn samples varying from 8.95 to 13.02% in total protein. They observed high correlations between protein content and the contents of each of these amino acids, but, contrary to expectation, were unable to detect any differences in the distribution of these amino acids between high- and low-protein corn. The observations of Frey ('51) are in harmony with the well-established fact that high-protein corn contains a greater proportion of zein in its total protein than does low-protein corn: tryptophan, absent in zein, and valine, in which zein is relatively deficient, occurred in decreasing proportions in the total protein of corn as the protein percentage increased.

The investigations on the chemical composition of corn and its modifying factors discussed above would lead one to expect a low nutritional value of zein, an expectation that has been amply realized by the investigations of Osborne ('13) and of Osborne and Mendel ('14). In fact, later experiments (Harris, Neuberger and Sanger, '43; Geiger and Hagerty, '49; Kligler and Krehl, '50) have been interpreted to mean that zein, even when supplemented with the essential amino acids in which it is deficient, will not support satisfactory growth, due to some hypothetical impaired digestibility, or "amino acid imbalance." The chemical data on corn would also lead one to expect that the nutritional quality of the proteins of high-protein corn is lower than that of low-protein. But this has not been proved. The experiments of Dobbins et al. ('50) with growing pigs indicated the validity



of this expectation, but unfortunately the number of pigs used was small and the variability among biological values was large so that no statistical difference could be established for corn containing 10.8 and 15.8% protein.

It will be the purpose of this report to test by biological means the expectations based upon the chemical composition of high- and low-protein corn with reference to possible differences in digestibility and biological value and the supplementary effects of tryptophan and of lysine in which zein is severely deficient. Growing albino rats have been used as experimental subjects.

EXPERIMENTAL METHODS

The experimental diets were all made according to the same pattern, the general features of which may be briefly described as follows: they were all complete in minerals and vitamins; all comparable diets were equalized in fiber at 2.0 or 2.25%, in fat at 5%, and in protein at levels varying in the different experiments. The corn components, consisting of entire kernels, varied in the different experiments; the lysine supplements when used were incorporated at a level of 0.25% and the tryptophan supplements at a level either of 0.20% or 0.40%. In one growth experiment a protein supplement consisting of two parts of meat scraps, two parts of soybean oil meal and one part of alfalfa meal was added to diets containing a constant proportion of corn of variable protein content, in amounts to equalize the protein content at about 18.5%. In all cases corn starch was used to complete the diets.

The nitrogen balance studies were carried out according to the usual procedure adopted in this laboratory, the essential features of which may be described briefly. Five or 6 pairs or trios of rats were carried through a series of experimental periods, each of which consisted of a preliminary feeding period of 7 to 10 days, followed by a collection period of 7 days, with constant food consumption during the preliminary and collection periods. Also, within each pair

or trio the food consumption was equalized throughout all periods. The general order of experimental periods may be illustrated by the order of diet testing followed in experiment 386:

	PERIOD I	PERIOD II	PERIOD III	PERIOD IV	PERIOD V
No. 1 rat in each pair	852	854	853	853s	852s
No. 2 rat in each pair	853	854	852	852s	853s

Diets 852 and 853 are the unsupplemented test diets, 852s and 853s are the corresponding diets supplemented with tryptophan and lysine, while diet 854 is the standardizing diet containing 4% of whole egg protein as the sole source of nitrogen. The nitrogen balance data secured in period II permit the calculation of the body's contribution to the feces (metabolic fecal N per gram of dry matter consumed) and to the urine (endogenous urinary N per $W_{gm}^{3/4}$). These nitrogen balance items are essential for the calculation of coefficients of true digestibility (T.D.) and biological values (B.V.) of the dietary nitrogen (protein). The formulas for these terminal calculations may be written as follows:

$$T.D. = \frac{N \text{ intake} - (\text{fecal N} - \text{metabolic fecal N})}{N \text{ intake}} \times 100$$

$$B.V. = \frac{N \text{ balance} + \text{endogenous N in urine} + \text{metabolic fecal N}}{N \text{ intake} - (\text{fecal N} - \text{metabolic fecal N})} \times 100$$

The numerator in the second equation epitomizes the uses to which the truly absorbed N is put in the body of the growing rat, i.e., nitrogen retention in growth and the replacement of body nitrogen losses in urine and feces in maintenance.

In the growth experiment referred to above, the empty carcasses of the rats at the termination of the experiment were analyzed for nitrogen and gross energy.

Diets, feces and urine collections, and carcasses were analyzed for nitrogen by the Kjeldahl method, using mercury as a catalyst and digesting, after clearing, for 4 hours in the case of diets, feces and carcasses, and for one hour in the case of urines. The gross energy (heat of combustion) of the

diet and carcass samples was determined with the oxygen-bomb calorimeter.

The corn samples were analyzed for total nitrogen and for nitrogen soluble in 71% ethanol, presumably zein nitrogen. They were also analyzed for tryptophan and lysine by microbiological methods. For tryptophan, hydrolysis of the protein was effected with 6 N Ba(OH)₂ by a modification of the method of Miller and Ruttinger ('50). The organism employed was *Streptococcus faecalis* R., and light transmission at 650 mμ was read in a Coleman spectrophotometer. For lysine determination, the samples were hydrolyzed with 2 N HCl for 5 hours at 15 lb. pressure. The organism employed was *Lactobacillus mesenteroides* P-60, following the procedure of Henderson and Snell ('48).

EXPERIMENTAL RESULTS

The chemical constituents in the corn samples submitted to nutritional evaluation are summarized in table 1. Particularly noteworthy are:

1. The increasing proportion of zein in the total protein as the protein content of the corn samples increases. In a much larger series of corn samples, 84, with protein contents ranging from 5.5 to 13.47%, the correlation coefficient was +0.95, and the regression of zein percentage in total protein (y) on total protein (x) was $y = -17.44 + 5.20x$. The regression departed from linearity at higher protein contents.

2. The decreasing percentage of tryptophan in the total protein (y) as the zein content of the total protein (x) increases. In the large set of data referred to above, this correlation is measured by a coefficient of -0.81, and a regression of $y = 1.126 - 0.0101x$.

3. The decreasing percentage of lysine in the total protein (y) as the zein content of the total protein (x) increases. For the large set of data, the correlation between these two variates is measured by a coefficient of -0.76, and a regression of $y = 4.029 - 0.0429x$.

The correlation and regression coefficients cited above are all highly significant statistically.

The nutritional evaluation of the proteins of the corn samples tested, with and without amino acid supplements, is given in table 2, together with statistical analyses of the differences between the average biological values in the three experiments.

TABLE 1
Pertinent constituents in corn samples tested

DESCRIPTION OF SAMPLES	PROTEIN (N \times 6.25) CONTENT ON DRY BASIS	ETHANOL- SOLUBLE IN TOTAL PROTEIN	TRYPTO- PHAN IN TOTAL PROTEIN	LYSINE IN TOTAL PROTEIN
	%	%	%	%
U.S. Hybrid 13, continuous corn, no soil treatment	7.32	23.2	0.87	2.92
U.S. Hybrid 13, corn, oats and clover rotation; lime, manure and rock phosphate fertilization	10.73	32.9	0.75	2.72
Ill. high protein grown on nitrogen- deficient soil	13.47	46.4	0.71	2.19
Ill. high protein grown on nitrogen- fertilized soil	20.04	57.7	0.55	1.76
Funk's Hybrid G94	9.23	45.1	0.76	2.19
Ill. high protein grown on nitrogen- fertilized soil	21.07	56.5	0.57	1.77

The digestibility of the nitrogen in all experiments averaged higher for the high-protein corn. The differences in digestibility were statistically significant at probability levels of 3% or less except in the comparison of diets 859 and 858 ($P = 0.09$) and diets 852 and 853. However, the digestibility of the nitrogen in diets 858s and 859s, averaging 94.8 and 95.9, respectively, differed significantly ($P < 0.0001$). These data are not given in table 2, because the biological values obtained on the supplemented diets were so erratic as to indicate clearly the operation of gross disturbing factors in

TABLE 2
The nutritive value of the proteins measured by nitrogen balance studies with growing rats

EXP. NO.	DIET NO.	CORN SAMPLES COMPARED AND THEIR PROTEIN CONTENTS ON THE DRY BASIS	AMINO ACID OR PROTEIN SUPPLEMENTS	PROTEIN CONTENT OF DIETS	NUMBER OF PAIRS USED IN EACH EX- PERIMENT	AVE. DIGESTI- BILITY OF PROTEIN	AVE. BIOLOGICAL VALUES OF PROTEIN	STATISTICAL COMPARISONS OF BIOLOGICAL VALUES		
								Diets compared	Mean difference	Student's t value
386	852	7.32%, U. S. hybrid 13 ¹	None	6.24	5	88.3	68.6	852 vs. 853	5.5	1.91
	853	10.73%, U. S. hybrid 13 ²	None	6.24		92.5	63.1	852 vs. 852s	10.4	3.72
	852s	Same as 852	0.25% DL-lysine and	6.31	in	88.5	79.0	853 vs. 853s	14.2	7.02
	853s	Same as 853	0.20% L-tryptophan	6.56	all	91.7	77.3	852s vs. 853s	1.7	0.93
446	858	13.47%, Ill. high protein ³	None	10.38	6	94.8	46.9	858 vs. 859	2.1	3.68
	859	20.04%, Ill. high protein ⁴	None	10.56	in all	95.3	44.7			0.0019
446a	864	9.23%, Funk's G94	None	8.06	6	93.1	56.8	864 vs. 865	7.6	5.08
	865	21.07%, Ill. high protein	None	7.88		95.4	49.2	864 vs. 864s	3.7	3.85
	864s	Same as 864	0.25 DL-lysine and	8.06	in	93.3	60.6	865 vs. 865s	3.8	2.13
	865s	Same as 865	0.40% DL-tryptophan	7.88	all	95.9	53.2	864s vs. 865s	7.4	4.54

¹ Grown on soil of low fertility, plot 3NE (Hamilton et al., '51).

² Grown on soil of high fertility, plot 5SW (Hamilton et al., '51).

³ Grown on a nitrogen-deficient soil.

⁴ Grown on a nitrogen-fertilized soil.

metabolism, but not in digestion. The preponderance of evidence clearly supports the conclusion that increased digestibility of the nitrogen accompanies an increased protein content of corn. For this small number of paired variates (6), the correlation coefficient is $+0.82$ and the regression of digestibility on protein content is $y = 88.0 + 0.3838x$. Both the correlation coefficient and the regression coefficient, 0.3838 , are significant at somewhat less than the 5% level of probability.

The biological values (y) of the protein of the various corn samples tested show an inverse relationship with the protein content (x), $r = -0.78$, $y = 73.59 - 1.372x$. However, neither r nor the regression coefficient, -1.372 , is significant statistically with $P = 0.077$. It is fair to consider that the statistical inadequacy is due to fewness and variability of data rather than to a possible independence of the variates, because of the established fact that the higher the protein content of corn, the greater the proportion of zein and the smaller the proportion of tryptophan and of lysine in the total protein.

In experiment 386, supplementation of the corn protein (U.S. 13) with tryptophan and lysine boosted the biological value of both low- and high-protein samples by 10 and 14 percentage points, respectively, increases that were highly significant statistically. Furthermore, the supplemented proteins, unlike the unsupplemented, possessed biological values that are indistinguishable statistically. This finding is in harmony with that of Marais and Smuts ('40), who reported an elevation of the biological value of corn, with a protein content of about 10%, from 67 to 81 by supplementation with tryptophan and lysine. Supplementation with either of the two amino acids alone had no appreciable effect on the biological value.

In experiment 446a, supplementation of both the low- and the high-protein corn with tryptophan and lysine raised the biological value of the protein, but only by somewhat less than 4 percentage units. Also, the supplemented corns show

the same difference in biological value as the unsupplemented, 7.4 and 7.6 percentage units, respectively. It should be noted, however, that unlike the case in experiment 386, the low- and high-protein corn, besides differing in protein content, differed also in variety and possibly also in the amino acid content of their proteins other than in tryptophan and lysine.

The growth experiment, no. 438, was designed to test the growth-promoting value of the protein in three corn samples containing 9.56, 15.80 and 19.69% protein on the dry basis, when supplemented with a protein concentrate mixture commonly used in swine feeding. The corn samples were incorporated in the diets at a constant percentage, 69.20, and the protein supplement, containing 45.31% of protein, at such levels as were required to raise the protein contents of the diets to approximately 18.5%.

Eleven trios of albino rats were employed in the test, with initial body weights ranging from 55 to 65 gm. One member of each trio was fed diet 830, one member diet 831 and the remaining member diet 832. The food intake of trio mates was equalized throughout the experiment, which lasted for 42 days. At the end of the feeding test, the rats were killed and the empty carcasses analyzed for protein ($N \times 6.25$) by the Kjeldahl method and the heat of combustion was determined with the oxygen-bomb calorimeter.

The main results of this experiment are summarized in table 3, with a statistical analysis of those diet differences that were the most significant when analyzed by the method of Student ('25).

Diets 830 and 831, containing corn samples with 9.56 and 15.80% protein, respectively, did not differ significantly in growth-promoting ability, as measured by body weight gains, by attained body length from nose to root of tail, or by protein deposition. The energy gain in the carcass was distinctly greater on the diet, 831, containing the corn sample with the greater content of protein.

Diets 831 and 832, containing corn components with 15.80 and 19.69% of protein, respectively, differed significantly in

TABLE 3
Results of the growth test, experiment 438

DIET NO.	PROTEIN IN CORN TESTED ¹	CORN IN DIET	PROTEIN IN SUPPLEMENT ²	SUPPLEMENT IN DIET	PROTEIN IN DIET	AVERAGE TOTAL BODY WEIGHT GAINS ³	AVERAGE BODY LENGTHS	AVERAGE PROTEIN IN CARCASS	AVERAGE ENERGY IN CARCASS
	%	%	%	%	%	gm	mm	gm	Cal.
830	9.56	69.20	45.31	26.55	18.25	171.3	211.1	44.27	582
831	15.80	69.20	45.31	19.11	18.56	171.0	210.4	42.88	629
832	19.69	69.20	45.31	12.05	18.69	164.5	208.9	40.90	602
			diets compared			831,832	831,832	831,832	830,831
			mean difference			6.45	1.5	1.98	47.1
			Student's P value			0.027	0.031	0.0048	0.0008

Statistical analysis of data:

¹ On the dry basis.

² Meat scraps 2, soybean oil meal 2, alfalfa meal 1.

³ Computed on the final empty body weight.

growth-promoting potency in favor of diet 831, as measured by body weight gains, attained body length after 42 days on feed, and protein deposition. The deposition of energy was greater on diet 831 by 27.4 cal., significant at the 4.2% level of probability.

DISCUSSION

This investigation, in conjunction with evidence published elsewhere, proves clearly that conditions, hereditary or environmental, that increase the protein content of corn, increase to a greater extent the content of the protein of poorest nutritional value, zein. Chemical and biological assays testify to the poorer nutritional quality of the protein of high-protein than of low-protein corn. The regression of percentage of zein in the total proteins of corn on total protein is such that, on the average and for protein contents up to 14% or thereabout, an increase of 1% in the total protein of corn will increase the proportion of zein by 5.2 percentage units on the average.

Biological tests of corn samples of greatly different protein content, using growing albino rats as subjects, reveal clearly, first, that the nitrogen of high-protein corn is more digestible than that of low-protein corn, the regression being such that each increase in the protein content of 1% is associated with an average decrease in the digestion coefficient of protein of 0.38 percentage units. The tests reveal further, in harmony with the chemical data, that increasing protein content is associated with decreasing biological utilization for maintenance and growth of the absorbed nitrogen. The regression is such that an increase of 1% in protein content of corn is associated with an average decrease of 1.37 in the biological value.

However, the low biological value of the proteins of corn, and the decreasing value as the protein content increases, is not a serious matter when corn is used in animal feeding. The suggestion of plant geneticists (Doty et al., '46; Frey, Brimhall and Sprague, '49) that the situation may be corrected by selective breeding of corn seems needlessly diffi-

cult. The easiest way and the most practical way is to use corn in farm rations in combinations with feeds capable of correcting the amino acid deficiencies of corn.

The content of corn proteins in the amino acids essential for growth reveals the fact that these proteins are limited in their biological utilization by a serious deficiency of lysine and of tryptophan (Mitchell and Block, '46). In this investigation it has been shown that supplementation of corn proteins with these two amino acids may boost the biological value by 10 to 14 percentage units, confirming earlier work by Marais and Smuts ('40), raising the biological value to a level comparable to that of meat protein.

At present it is impractical to correct the amino acid deficiencies of corn by pure amino acids, but it has been known for some time that the desired result can be secured by feeding corn in combination with feed proteins relatively rich in tryptophan and lysine. One of the first demonstrations of this possibility was reported by Hart, Steenbock and Letcher ('22), who showed that corn proteins when combined with milk proteins in the proportion of 1 to 0.4 yield a protein mixture which is highly efficient in promoting nitrogen retention in growing pigs. Calculations from their data (Mitchell, '24) show that the biological value of the proteins of such a mixture is about 87, compared to 60 for corn proteins.

Dobbins et al. ('50) tested the supplementary relations for growing pigs of a mixture of equal parts by weight of alfalfa meal, soybean meal and meat and bone scraps added to rations containing corn varying in protein content from 8.2 to 12.8%. When the ratio of corn N to supplemental N remained above 1.00, the protein mixtures were equally efficient in promoting growth. Only when for the rations containing the highest protein corn the ratio fell to 1:0.57, and, for heavier body weights, to 1:0, was the inferior nutritional value of the corn containing 12.8% protein evident.

In the growth test with rats of the investigation here reported, much the same supplemental protein mixture was

used as that employed by Dobbins et al. Here it was shown that for corn containing 15.8% protein, a ratio of corn N to supplemental N of 1:1 (diet 831) was as good nutritionally, within the limitations of the technic employed, as a ratio of 1:1.85 (diet 830) with corn containing 9.56% protein. However, in diet 832, containing corn with 19.69% protein and a ratio of corn N to supplemental N of 1:0.435, a depression in growth and protein storage was revealed.

The proteins of soybeans, meat scraps and alfalfa meal are not deficient nutritionally in either tryptophan or lysine; the mixture used in this investigation contained about 1.16% tryptophan and 5.9% lysine on the crude protein content, according to calculations based on data reported by Stokes et al. ('45) and Block and Bolling ('51). Apparently when such a mixture is fed with corn of a protein content as high as 16%, so that the ratio of corn N to supplemental N does not fall much below 1:1, the nutritive quality of the mixture is not appreciably different from that resulting from a mixture of average protein corn (9.5%) and supplement in which the protein ratio is much higher, 1:1.85.

CONCLUSIONS

The proportion of zein in the total proteins of corn increases linearly with the total protein content up to a content of about 14%. At higher protein levels the regression apparently departs from linearity. Within the limits indicated the correlation of the two variates is high with $r = +0.95$.

The proportions of tryptophan and of lysine in the total proteins of corn decrease with increasing content of protein.

The digestibility of the mixed proteins of corn increases slightly as the protein content of corn increases, but the biological value decreases considerably. Supplementation of the proteins of corn with lysine and tryptophan will raise the biological value to levels that may approximate the biological value of meat protein ($N \times 6.25$).

Effective supplementation, even of the proteins of the highest-protein corn tested, may be attained with protein mixtures commonly used in swine feeding. Hence, the lowered biological value of the proteins of high-protein corn is not a detriment to their proper use in swine, and presumably poultry, feeding, and, within limits of protein content undefined at present, will spare the use of protein supplements.

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THE INFLUENCE OF VITAMIN B₁₂ AND ANTIBIOTICS ON PROTEIN AND ENERGY UTILIZATION IN A LOW PROTEIN DIET^{1, 2}

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It is well known that vitamin B₁₂ is associated with growth in many species of animals, and it is likewise recognized that the addition of antibiotics to the rations of some of these animals, such as chickens, turkeys, and swine, produces a further growth response over that obtained with vitamin B₁₂ alone in a basal ration which is otherwise complete except for these supplements. Since a stimulation of growth is involved in both of these circumstances, there is suggested the possibility that vitamin B₁₂ and antibiotics are involved in protein metabolism.

In experiments designed to study this question, Hartman, Dryden and Cary ('49) observed that a deficiency of vitamin B₁₂ may have a very deleterious effect upon the growth of rats. From this work they concluded that vitamin B₁₂ must play a fundamental role in affecting the capacity of normal mammals to utilize protein. Hove and Hardin ('51) found that rats which were consuming a 10% protein diet gained more weight per gram of protein ingested when vitamin B₁₂ was included in the diet than when it was omitted, which

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indicated to them that efficiency of protein utilization was influenced by vitamin B₁₂. Further, it was shown by Charkey, Wilgus, Patton and Gassner ('50) that vitamin B₁₂ has a possible function in utilization of circulatory amino acids, since a higher level of amino acids was found in the blood stream of B₁₂-deficient chicks than in B₁₂-supplemented birds.

Henry and Kon ('51) found the biological value of casein determined with rats to be higher when supplemented with vitamin B₁₂ than when vitamin B₁₂ was missing, but true digestibility was unaffected by the vitamin. Although their results demonstrate that the assimilation of nitrogen was less in the absence of vitamin B₁₂ than with it, the authors were of the opinion that this relationship was not necessarily specific.

Chow and Barrows ('50) concluded from work with rats that carbohydrate or fat metabolism was more affected by vitamin B₁₂ than was protein metabolism, since they found vitamin B₁₂ to be without influence on nitrogen retention.

In recent work from this laboratory Black and Bratzler ('52) studied the effect of vitamin B₁₂ and antibiotics, singly and in combination, on protein and energy metabolism in rats, and found that protein utilization, as such, was not differentially affected by these supplements when the level of protein in the diet was 18%. In these experiments significantly greater body gains were observed with rats when vitamin B₁₂ was added to a diet of low B₁₂ content than when it was omitted, but indications were lacking that protein, specifically, was more efficiently utilized than was fat or total gain of energy.

The present experiment was designed to study this problem further by investigating the combined effects of vitamin B₁₂ and certain antibiotics on protein and energy utilization in the rat when a low protein diet was fed.

EXPERIMENTAL

This experiment, like the previous one (Black and Bratzler, '52), involved a 70-day growth and metabolism investigation

with albino rats, employing the body balance techniques described by Swift et al. ('34).

The experimental subjects consisted of two groups of 12 litter-mate pairs of albino rats, each animal of a pair being of the same sex and approximately the same weight at the start of the experiment. The distribution of animals according to sex was nearly equal; 7 pairs of males and 5 pairs of females were used. The diets (table 1) for the two

TABLE 1
Composition of diets

	UNSUPPLEMENTED	SUPPLEMENTED
	%	%
Soybean oil meal (solvent)	16.78	16.78
Linseed oil meal (solvent)	6.84	6.84
Salt mixture IV ¹	4.00	4.00
Mazola	5.00	5.00
Dextrin	33.69	33.69
Cerelose	33.69	33.69
Vitamin B ₁₂ supplement ²		0.50
Aureomycin hydrochloride		0.0047
Streptomycin sulfate		0.0047

¹ Lichstein et al. ('46).

² The vitamin B₁₂ supplement was Bi-con 3*, a product of Chas. Pfizer and Co.

Vitamins were added in the following proportions on a kilogram basis: Oleum percomorphum, 570 mg; tocopherols (mixed) 150 mg; choline, 4,000 mg; inositol, 2.0 gm; folic acid, 0.25 µg; biotin, 0.10 µg; thiamine, 5 mg; riboflavin, 5 mg; pyridoxine hydrochloride, 6.25 mg; niacin, 6.25 mg; calcium pantothenate, 50 mg; para-aminobenzoic acid, 95 mg; 2-methyl 1,4-naphthoquinone, 2.5 mg.

groups of animals were identical, except that to one diet there was added a vitamin B₁₂ supplement in a quantity sufficient to supply 33 µg of vitamin B₁₂ activity per kilogram of diet and 5.5 mg of antibiotic in the form of streptomycin and terramycin per kilogram. Streptomycin sulfate and aureomycin hydrochloride were also added to the supplemented diet in equal quantities so that there were 100 mg of total antibiotic per kilogram of diet.

The rats were fed in accordance with the paired-feeding technique, which involved limiting the amount of feed of

one animal to the amount consumed by the member of the pair eating the least.

In order to compare the feed intake and growth obtained with the paired-feeding technique with those obtained when the feeding was on an ad libitum basis, another group of 8 rats were fed the supplemented diet ad libitum for a 5-week period. Observations in this experiment were limited to only feed intake and growth.

RESULTS AND DISCUSSION

It was evident in the paired-feeding experiment that the rats receiving the unsupplemented diet determined the feed consumption, since of a total of 840 feed offerings there were 436 refusals of feed in the unsupplemented group and only 69 refusals in the supplemented group.

The average amounts of feed eaten, the gains in body nitrogen and fat, and the live weight are given in table 2. Some of these data for the pair-fed group are given both for a 5-week and a 10-week period so that a comparison can be made with the ad libitum-fed animals that were on experiment only 5 weeks.

The pair-fed rats that received the basal diet gained an average of 122 gm in weight, while those on the supplemented diet gained 127 gm in weight. The statistical significance of such a difference, when computed by Student's method as modified by Love ('24), shows odds of 83 to 1 that the difference did not occur by chance alone. Most of the difference in gain in body weight between the two groups was accounted for by gain of fat—the greater amount being in favor of the animals that received the supplemented diet. Odds indicating the significance of this difference were 51 to 1. Thus there was an indication that not only did the supplemented animals gain more weight, but that this gain was of higher energy content. A further observation regarding body composition was the water content; however, these values are not shown in table 2. For the rats on the basal diet the bodies contained 63.7% water and on the supplemented

TABLE 2
Average amounts of feed eaten and gains in live weight, nitrogen and fat

DIET	TIME ON EXP.	METHOD OF FEEDING	FEED EATEN	LIVE WEIGHT		GAIN IN LIVE WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED
				Initial	Final			
			<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
Unsupplemented	5	Paired	292 ± 7 ¹	47 ± 1.1	110 ± 2.3	63 ± 2.1		
Supplemented	5	Paired	292 ± 7	47 ± 1.0	115 ± 2.6	68 ± 2.4		
Unsupplemented	10	Paired	672 ± 14	47 ± 1.1	169 ± 3.9	122 ± 3.8	3.9 ± 0.1	14.6 ± 0.8
Supplemented	10	Paired	672 ± 14	47 ± 1.0	174 ± 4.4	127 ± 4.2	3.9 ± 0.1	18.2 ± 1.5
Unsupplemented	5	Ad libitum	300 ± 8	47 ± 1.1	118 ± 4.0	71 ± 3.9		

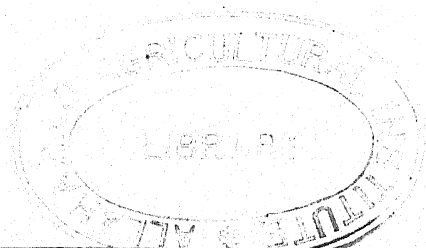
¹ Standard error of the mean.

diet this value was 62.5%. In the ad libitum-fed rats the live weight was 118 gm at the end of 5 weeks, compared to 110 gm and 115 gm for the pair-fed rats on the control and supplemented diets, respectively, for the same length of time. It was expected that the ad libitum-fed rats would consume more feed than the pair-fed rats, since feed consumption was limited largely in the pair-fed rats by those on the control ration, but at the end of the 5-week experimental period, differences in feed consumption and weight gains between the two supplemented groups fed by the different feeding techniques were very small (table 2). Obviously the amount of feed consumed by the unsupplemented group in the pair-fed experiment was extremely close to the amount which would be consumed by the supplemented group as a maximum. A slight variability may have been due to the use of different animals, but the magnitude of the difference is sufficiently small to appear unimportant.

Black and Bratzler ('52), using the same type of diet and supplement employed here, but with an 18% protein level, obtained quite significant weight differences in rats on unsupplemented and supplemented diets. The small differences in weight gains obtained in the present work are attributed mainly to the suboptimum level of protein used. This low protein level appears to limit any beneficial effects that the added vitamin B₁₂ or antibiotics may produce. It is emphasized that considerably less feed is consumed on such low-protein diets, resulting in a disproportionately smaller amount of feed available for body gain.

The average distribution of feed nitrogen as affected by the vitamin B₁₂ and antibiotic supplementation is given in table 3.

Body gains of nitrogen determined either by difference, as in table 3, or by direct analysis as in table 2, were practically identical in both groups, so at the level of protein used neither vitamin B₁₂ nor antibiotics functioned by increasing protein utilization.



In other experiments where the diet contained 18% protein and feeding was on an ad libitum basis, Black and Bratzler ('52) found a much greater assimilation of nitrogen when vitamin B₁₂ was present in the ration than when it was deficient; however, they did not find this relationship to be specific for protein. Gains of fat and total gain of energy were similarly affected. They also found that when the feed intake was on a pair-feeding basis and the protein intake still at the 18% level, neither assimilation nor utilization of nitrogen was affected by vitamin B₁₂.

TABLE 3
*Distribution of feed nitrogen as affected by vitamin B₁₂ and
antibiotic supplementation*

DIET	UNSUPPLEMENTED	SUPPLEMENTED
Nitrogen		
Food, gm	10.96 ± 0.2 ¹	10.96 ± 0.2
Feces, gm	2.11 ± 0.0	1.99 ± 0.0
Digested, gm	8.85	8.97
% of food N	80.8 ± 0.3	81.9 ± 0.5
Urine, gm	4.54	4.85
% of food N	41.4 ± 0.7	44.4 ± 0.7
Gain of N, gm	3.88 ± 0.1	3.91 ± 0.1

¹ Standard error of the mean.

A comparison of the data of the present experiment with those of Charkey et al. ('50) cannot be made on a direct basis, since the level of protein in the diets was very different and excretion of nitrogen was not determined by Charkey and co-workers. It is interesting to note, however, that Charkey postulated that there was less renal loss in their experiment in the vitamin B₁₂-supplemented chicks than in those without this supplement in their diet. In the present experiment renal loss was measured and was greater in the rats receiving the vitamin B₁₂ and antibiotics than in the controls. Charkey et al. ('50) were of the opinion that the lowered amino acid content of the blood and the greater body gain of the vitamin B₁₂-supplemented chicks were associated with a lowered renal wastage.

The method used by Hove and Hardin ('51) in calculating protein utilization is not entirely satisfactory, since they assume that all gain in weight is of equal composition. Some or all of the increased weight gains could have consisted of fat or water. While the present work is in complete agreement with that of Hove and Hardin and that of Hartman, Dryden and Cary ('49) as far as the influence of vitamin B₁₂ on growth is concerned, it lacks specific indication that vitamin B₁₂ has a greater influence on protein metabolism than on fat metabolism or simply on total gain of energy. In this

TABLE 4
Distribution of total feed energy as affected by vitamin B₁₂ and antibiotic supplementation

DIET	UNSUPPLEMENTED	SUPPLEMENTED
Energy value in Cal.		
Food	2,623 ± 55 ¹	2,623 ± 55
Feces	221 ± 5	265 ± 8
Digested	2,401 ± 50	2,358 ± 49
Urine	55 ± 2	46 ± 1
Metabolized	2,347 ± 49	2,312 ± 48
Body gain: total	284 ± 12	318 ± 14
as protein	148 ± 5	133 ± 7
as fat	136 ± 8	185 ± 12
Heat production	2,063 ± 41	1,994 ± 41

¹ Standard error of the mean.

respect the conclusion of Henry and Kon ('51) that vitamin B₁₂ lacks specificity in regard to protein utilization appears to be the most logical, but further experimentation of a somewhat different nature than that so far reported will be required to show clearly the role of vitamin B₁₂ in the building of body tissue.

From the distribution of total feed energy as given in table 4 it is shown that the energy of the feces of the unsupplemented animals was less than that of the feces of the supplemented animals, resulting in a higher digestible energy for the control group, this being highly significant, as is shown by odds of more than 10,000 to 1. The magnitude of

this difference in digestible energy is reflected almost directly in the metabolizable energy, as the urinary energy is very similar in both groups. Consequently, the control rats had a significantly higher amount of metabolizable energy available, as shown by odds of 10,000 to 1, but in spite of the greater metabolizable energy available, the body gain of energy was less in this group. In the unsupplemented animals 10.8% of the feed energy went into body gain as compared to 12.2% for the supplemented animals, with most of the difference in body gain of energy being represented by the larger gain of energy as fat. The significance of the difference in energy gained as fat is shown by odds of 494 to 1. This observation on gain of fat is in confirmation of earlier work in which a similar method of feeding was employed (Black and Bratzler, '52).

The final difference observed in the partition of energy was with respect to heat production. The unsupplemented animals used 78.7% of the feed energy for heat production, which was significantly higher than the 76.0% of the feed energy used by the supplemented animals for the same purpose (odds 10,000 to 1).

Since heat production is a waste of feed energy insofar as growth is concerned, the vitamin B₁₂ and antibiotic supplements evidently were somewhat more effective in conserving some of this energy for body gain than was the unsupplemented diet. It is interesting to note that even though metabolizable energy was greater in the unsupplemented rats, body gain of energy was higher in the supplemented animals. This can mean that once the feed energy is absorbed from the intestinal tract the supplements play a role in the utilization of this energy for body gain.

In line with this thought is the postulation that antibiotics have an effect on the intestinal flora of the rat by favoring the production of those bacteria which can synthesize vitamin B₁₂, and, at the same time, suppressing the growth of certain microorganisms that utilize vitamin B₁₂ for their

growth and thus make it unavailable to the rat (Cravioto-Monoz et al., '51).

Much work has been done showing that vitamin B₁₂ and various antibiotics influence growth in many animals, but the exact mechanism by which this is accomplished is still unknown. It is thought, however, that increased feed consumption induced by the supplements is one of the major factors involved in this process. Also, in this work it has been shown that food is better utilized after absorption from the intestinal tract when vitamin B₁₂ and antibiotics are present than when these supplements are absent.

SUMMARY

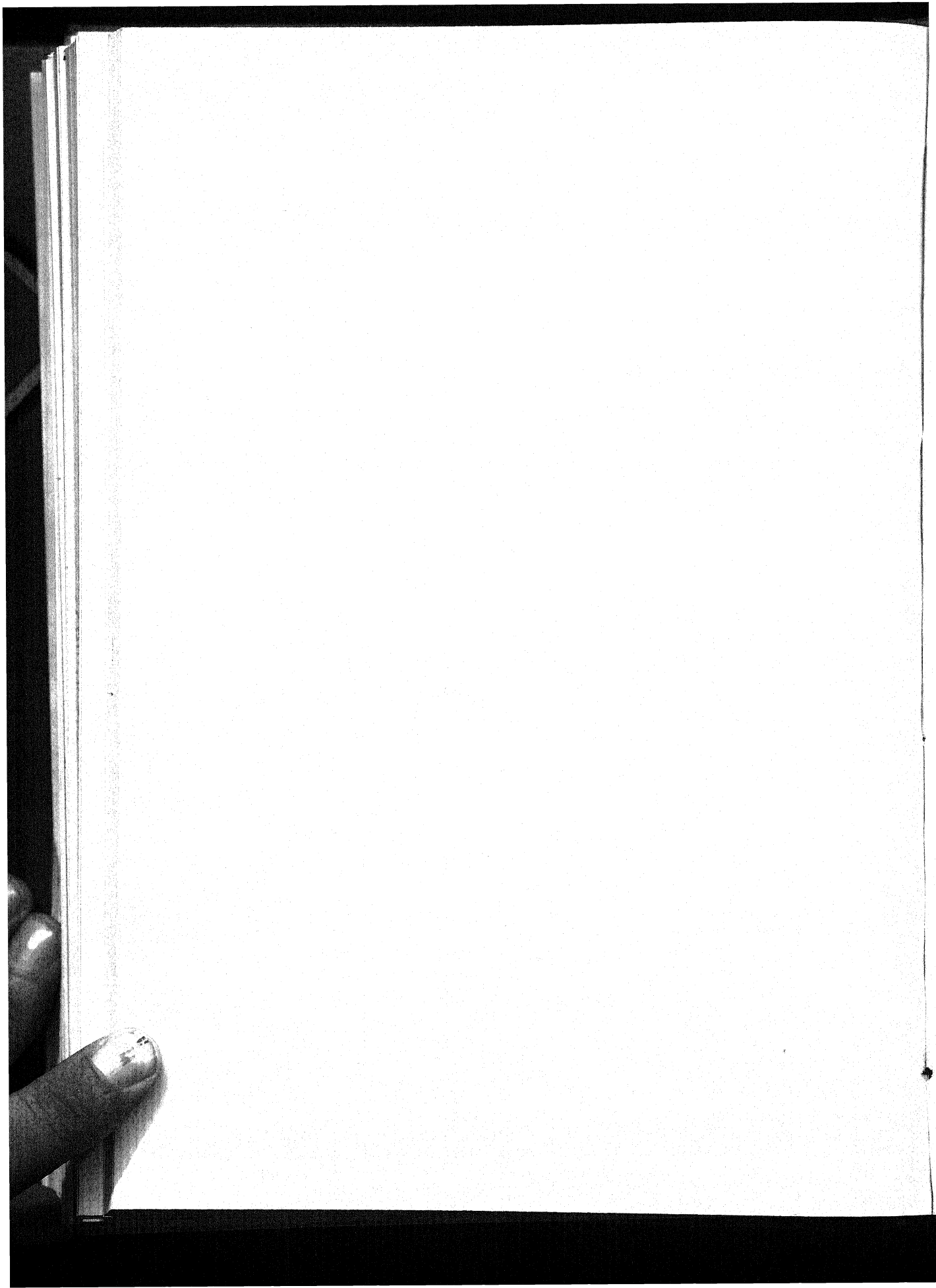
The effects of a combination of vitamin B₁₂ and antibiotics on protein and energy utilization were investigated in a 70-day growth and body balance experiment with 12 litter-mate pairs of growing albino rats. One animal of each pair was fed an unsupplemented diet compounded to contain adequate quantities of all known essential nutrients except vitamin B₁₂, while its litter mate was fed the same diet, in equivalent amounts (paired-feeding), plus a supplement containing vitamin B₁₂ and antibiotics. The protein in the diet was of vegetable origin and was fed at a 10% level.

The rats on the supplemented diet gained somewhat more live weight, stored more fat and less water, had less digestible and metabolizable energy and produced less heat than the animals on the unsupplemented diet.

At the level of protein fed in this experiment there was no indication that a specific relationship existed between the utilization of nitrogen and the supplements used, which is in confirmation of previous work in which a higher level of protein was fed. The utilization of energy-producing nutrient absorbed from the intestinal tract was shown to be improved by the presence of vitamin B₁₂ and antibiotics in the diet.

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CHRONIC SODIUM CHLORIDE TOXICITY IN THE ALBINO RAT

I. GROWTH ON A PURIFIED DIET CONTAINING VARIOUS LEVELS OF SODIUM CHLORIDE

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The importance of dietary salt has been recognized from time immemorial, and dietary sodium has been suspected for over 30 years as a primary cause of arterial hypertension in man (Allen and Sherrill, '22); yet little evidence has accumulated on the chronic toxicity of sodium chloride. The more primitive type of renal glomerulus in fowl as compared to the mammal makes it more susceptible to sodium chloride poisoning; hence most studies have been made on birds (French, '35; Krakower and Goettsch, '45; Lenel, Katz and Rodbard, '48). Campbell ('46) reported on the growth and reproduction of the rat on diets containing 1.32, 2.59 and 5.06% of sodium chloride. No great differences were observed in growth, apparent well-being or ability to reproduce. There were some indications of renal damage at the highest level of sodium chloride intake. Meyer et al. ('50) found that high level feeding of sodium retarded somewhat the growth of rats.

This report covers the growth, ration consumption, early mortality, and general observations on rats fed a purified diet containing sodium chloride at 7 different levels.

EXPERIMENTAL

Ration I contained casein, vitamin-test,¹ 25.0%; cane sugar, 51.9%; shortening, all-vegetable (Crisco), 20.0%; a mineral mixture,² 2.9%; and a vitamin mixture,³ 0.2%. It is a low-sodium diet (no sodium chloride added but containing 0.01% of sodium chloride by analysis). Rations II to VII, inclusive, were made by intimately mixing ration I with the calculated amount of sodium chloride, C. P. powder, in a Hobart mixer. Ration II contained an adequate reasonable amount of sodium chloride equivalent to incorporating the Hubbell, Mendel and Wakeman ('37) salt mixture at about a 2.0% level. Rations III to VII, inclusive, were progressively higher in sodium chloride content, 20, 40, 50, 60 and 70 times the Hubbell, Mendel and Wakeman formula fed at a 2% level. Rations were made fresh at no more than 15-day intervals and were stored at 7°C. until placed in feed cups.

Male, Sprague-Dawley, albino rats, 33 to 40 days old and weighing from 86 to 154 gm, were divided into 7 matched groups and placed on demineralized water and the purified diets ad libitum. The rats were kept in steel wire cages (several rats per cage) in a constant temperature room ($27^{\circ} \pm 2^{\circ}\text{C}.$). They were fed, watered and observed daily, and individually weighed and carefully examined weekly. The rate of food and water consumption was studied for the first 5 weeks of the experimental regimen. Scatter-proof feed

¹ Manufactured by Nutritional Biochemicals, Inc., Cleveland, Ohio.

² The mineral mixture is a modification of the Hubbell, Mendel and Wakeman ('37) formula by weight, as follows: CaCO_3 , A. R., 543.0; KH_2PO_4 , A. R., 212.0; KCl , A. R., 112.0; FePO_4 , N. F., 38.1; MgCO_3 , A. R., 25.0; MgSO_4 , A. R., 16.0; $\text{KF} \cdot 2\text{H}_2\text{O}$, A. R., 2.20; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, A. R., 1.41; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, U. S. P., 0.90; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, A. R., 0.39; $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, U.S.P., 0.31; KI , C. P., 0.08; and $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, C. P., 0.01.

³ The vitamin supplementation in weight per kilogram of ration I was as follows: thiamine hydrochloride, 5.0 mg; riboflavin, 5.0 mg; pyridoxine hydrochloride, 2.5 mg; vitamin B_{12} , 40.0 μg ; *l*-inositol, 1.0 gm; *p*-aminobenzoic acid, 2.5 mg; biotin 24.0 μg ; niacin, 20.0 mg; calcium pantothenate, 30.0 mg; choline chloride, 1.0 gm; menadione bisulfite, 4.8 mg; α -tocopherol, 30.0 mg; folic acid, 1.0 mg; and concentrated oleovitamin A and D, U.S.P., 0.4 gm.

cups ⁴ and inverted wide mouth bottles with stainless steel water feeder tubes ⁵ were used.

RESULTS

Growth

Table 1 lists the per cent of sodium chloride in the ration and the group means and standard deviations of the gross body weight fortnightly for the first 20 weeks of the experimental regimen. This tabulation includes only those rats which exhibited good health throughout the test period. Excluded here were some 11% of the rats which developed abnormalities such as intercurrent infection or which sustained injuries and were removed from the colony. This omission was made in order to keep the number of animals in each group constant throughout the 20-week period. However, the growth pattern of the excluded animals did not differ from that of their respective group until edema or other diseased states intervened. A separate report is to be made on the pathologic changes observed in these animals (Meneely et al., '52).

Food consumption

Table 2 lists the rate of food consumption during the first 5 weeks of the experimental regimen. The values are the weekly means expressed as grams of food per rat per day. These values represent an over-estimation of the amount of food eaten by the rats, because some undetermined wastage occurred. The semi-plastic consistency of the food and the scatter-proof feed cups tended to reduce the food wastage to a minimum.

Water consumption

Table 3 lists the rate of water consumption during the first 5 weeks of the experimental regimen. The values are the

⁴ Manufactured by C. H. Sommers and Associates, Cincinnati, Ohio.

⁵ See footnote 4.

TABLE 1
Growth data (gross body weight in grams at bi-weekly intervals)

TIME IN WEEKS	RATION I 0.01% NaCl		RATION II 0.15% NaCl		RATION III 2.8% NaCl		RATION IV 5.6% NaCl		RATION V 7.0% NaCl		RATION VI 8.4% NaCl		RATION VII 9.8% NaCl	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Initial	116	19	119	17	118	17	117	19	121	17	118	21	121	22
2	170	30	184	26	179	18	176	21	180	21	176	20	173	21
4	207	41	251	28	244	24	229	19	244	22	237	22	228	21
6	226	45	296	29	297	28	276	22	290	28	277	27	269	23
8	256	44	334	31	330	28	315	24	322	30	313	29	301	27
10	281	49	360	32	354	30	339	24	341	31	334	31	324	25
12	315	58	392	34	380	29	365	23	365	32	353	29	341	25
14	340	55	412	36	393	30	374	24	377	33	367	30	349	24
16	359	60	432	38	412	33	394	24	396	35	385	36	368	28
18	388	56	454	38	435	34	414	27	411	35	399	37	383	26
20	416	43	472	39	449	34	432	26	424	36	413	35	395	28
N	10		30		29		28		24		26		25	

weekly means expressed as milliliters of water per rat per day. These values also represent some over-estimation of the water drunk by the rats as an unavoidable amount of spillage occurred.

TABLE 2

Mean ration consumption. All figures are in grams per rat per day

RATION	I	II	III	IV	V	VI	VII
% NaCl	0.01	0.15	2.8	5.6	7.0	8.4	9.8
1st Week	11.1	11.4	11.4	11.5	12.6	12.3	11.7
2nd Week	10.3	12.4	12.8	12.9	14.0	13.7	13.3
3rd Week	10.5	13.4	13.3	13.5	13.8	14.3	13.9
4th Week	10.4	14.2	13.7	12.7	14.7	15.6	15.0
5th Week	10.2	13.9	14.0	14.8	14.6	14.6	13.9
Mean	10.5	13.1	13.0	13.1	13.9	14.1	13.6

TABLE 3

Mean water consumption. All figures are in milliliters per rat per day

RATION	I	II	III	IV	V	VI	VII
% NaCl	0.01	0.15	2.8	5.6	7.0	8.4	9.8
1st Week	24.0	26.4	47.9	55.7	60.0	72.1	73.4
2nd Week	28.6	30.2	40.6	63.4	71.9	77.8	84.9
3rd Week	29.4	38.9	46.1	65.8	76.1	84.0	90.0
4th Week	30.8	45.2	58.0	65.1	82.9	102.7	91.2
5th Week	25.0	49.0	63.1	74.3	87.7	104.1	89.2
Mean	27.6	37.9	51.1	64.9	75.7	88.1	85.7

Mortality and morbidity

The rats grew well on all of the experimental rations. Only 17 rats out of a total of 193 died or were sacrificed during the 20-week period covered in this report. Table 4 summarizes the mortality data. The morbidity included two rats on ration IV with mechanical injury to the snout, one rat on ration V which contracted respiratory infection and one rat on ration VI which developed edema.

DISCUSSION

The diet herein described is of known composition, is reproducible from available, chemically pure nutrients, and is of a desirable consistency for feeding to small animals. It is very low in cholesterol, the only source being the concentrated oleovitamin A and D oil. The diet contains adequate amounts of all vitamins which are known to be required by the rat. In addition a few vitamins are incorporated of which, presumably, the rat does not require a dietary source, the reason being that sufficiently long-term studies have not been

TABLE 4
Mortality experienced during the first 20 weeks on the purified rations

RATION	CAUSE OF DEATH	NUMBER
I	Respiratory infection	1
II	Fractured nose	1
III	Respiratory infection	1
IV		0
V	Renal failure with edema	2
	Respiratory infection	2
	Fractured nose	1
VI	Renal failure with edema	2
	Respiratory infection	1
VII	Renal failure with edema	4
	Renal failure without edema	1
	Accidental hemorrhage	1

reported in every case, and it was deemed desirable not to risk the occurrence of vitamin deficiencies in the present long-term study.

The sodium chloride added to the rations was in a powdered form, freshly screened to render it uniform, and adequately mixed into the diet at high speed in a Hobart mixer. The rat had no chance to reject the added salt from his diet. The dispersion was such that if the rat ate any of the ration, he was forced to ingest the calculated fraction of sodium chloride.

The low-sodium diet (ration I) caused mild anorexia. The small group of rats on this diet ate less, drank less water,

and grew more slowly than did the controls (ration II). These animals exhibited a continuous salt hunger. This was manifest by their habit of licking any object in the cage, particularly wherever fingerprints were left. There was considerable variation in the rate of growth of the animals on ration I as demonstrated by the large standard deviations in gross body weight (table 1).

The high sodium chloride diets (rations III to VII, inclusive) depressed the rate of growth. Table 5 indicates the significance of the differences between the means of the control animals and the other 6 groups. Although the data on

TABLE 5

The probability (P) that the mean body weight of the sample differs from the mean body weight of the controls (ration II). Values in italics indicate that the difference is statistically significant

RATION	START	4 WEEKS	8 WEEKS	12 WEEKS	16 WEEKS	20 WEEKS
I	> .6	< .01	< .01	< .01	< .01	< .01
III	> .6	> .3	> .4	> .1	< .05	< .02
IV	> .5	< .01	< .01	< .01	< .01	< .01
V	> .8	> .3	> .1	< .01	< .02	< .01
VI	> .8	< .05	< .01	< .01	< .01	< .01
VII	> .6	< .01	< .01	< .01	< .01	< .01

ration consumption indicate that the rats on high salt diets ate as much or more than the controls, they did not consume as many calories as did the control animals and, consequently, did not gain as rapidly in gross body weight. The fact that most of the rats on the high salt diets grew and thrived demonstrates the remarkable ability of the rat kidney to excrete enormous quantities of salt. As anticipated, these rats exhibited a marked polydipsia and polyuria.

Although food and water spillage by the rat introduces some error into the data on ration consumption (tables 2 and 3), the relative values are reasonably correct. Trends are apparent from a casual inspection of the data. There can be little doubt that the low salt regimen had a depressing

effect on the appetite and that the high salt diets caused thirst but did not depress the appetite to a marked degree.

The general appearance of the rats on the 7 purified diets was good throughout the test period with the following exception. When first placed on the high salt diets, many of the animals developed a condition of extreme dehydration characterized by marked porphyrin staining of the hair and extremities. This occurred during the first week of the experiment. By the end of the second week the condition had almost disappeared and did not recur. The rats had successfully adapted to the high-sodium diet.

Of the 17 deaths occurring during the 20-week period (table 4) only 9 were directly attributable to dietary stress. Less than 5% of the total number of animals involved succumbed to pathologic changes induced by sodium chloride toxicity or 10% of the animals on the three higher levels of sodium intake.

Sporadic renal damage was manifest by the sudden appearance of massive edema. The extracellular fluid measured with radioactive sodium-24 rose from control values of $26.2 \pm 1.88\%$ of body weight to 57.3 and 57.8% of body weight in two rats. During the week of most rapid edema formation, body weight gains averaged 62.8 gm for 9 edematous rats in contrast to an average gain of 10.1 gm a fortnight earlier for the same animals. The edematous rats were characterized by marked anemia, hypoproteinemia, lipemia, and azotemia. Histologic examination of the kidneys revealed extensive glomerular and tubular lesions. The pathology of these rats will be reported when the study is completed.

The surviving animals on all 7 rations were of uniform, healthy appearance indistinguishable with respect to fur, skin, color and behavior when handled from controls fed on Purina Chow. The size of the animals was a notable exception.⁶

⁶ The control rats on ration II actually exceeded the Purina Chow-fed rats in rate of growth. However, the test was not legitimate, because the Chow-fed rats were the extremes in weight rejected from the sample at the start of the experiment, and the data are omitted specifically for that reason.

The rate of growth on ration II (controls) compares favorably with other reports in the literature (Copping et al., '51; Freudenberger, '32; Mendel and Hubbell, '35; Zucker et al., '41). However, the "rapidly growing" rats of Pickens et al. ('40), and the small group of rats raised on a purified diet by Mayer ('48) grew more rapidly than did our control animals.

SUMMARY

Young male rats have been raised on a purified diet, containing 7 different levels of sodium chloride, and water ad libitum with the following results:

Rats fed diets high (2.8 to 9.8%) and low (0.01%) in sodium chloride grew more slowly than did rats on a control ration (0.15% NaCl).

High sodium chloride feeding caused polydipsia and polyuria but did not have a marked effect on appetite. A low-sodium diet caused mild anorexia.

Edema developed during this first 20-week period in 10% of the rats fed diets containing from 7.0 to 9.8% of sodium chloride. The majority of the animals (89%) remained in excellent health throughout the 20 weeks.

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THE METHIONINE REQUIREMENT FOR THE GROWTH OF SWINE

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The report of Bell et al. ('50) showed that methionine is an essential amino acid for the growth of swine. The authors suggested that on a diet containing 10% protein, the methionine requirement for swine is between 0.07 and 0.27% of the ration. Later Shelton et al. ('51) reported excellent growth of pigs on a 21% protein ration containing oxidized casein and gelatin plus tryptophan, and supplemented to contain 0.6% methionine. They demonstrated further that 0.3% of L-cystine could replace one-half of the methionine. More recently Curtin et al. ('52) showed that when weanling pigs were fed a soybean oil meal-purified ration containing 22% protein the methionine requirement did not exceed 0.37% in the presence of 0.38% cystine.

In the present work, a methionine deficient ration containing isolated soybean protein² and brewers' dried yeast was fed to weanling pigs in two experiments to obtain additional data on the methionine requirement of swine. The studies of Grau and Almquist ('43) demonstrated that isolated soybean protein is deficient in the sulfur containing amino acids for optimum chick growth. Likewise, brewers' dried yeast is low in methionine (Block and Mitchell, '46-'47).

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² "Alpha protein" produced commercially by the Glidden Co., Chicago, Ill.

PROCEDURE

The basal rations fed are shown in table 1. No choline chloride was added to the ration since it has been shown that choline has a sparing effect on the methionine requirement for swine (Dyer et al., '49). Ration A was fed in the first trial

TABLE 1
Composition of basal rations

CONSTITUENTS	A	B
Isolated soybean protein ¹	21.3	
Isolated soybean protein, washed ²		20.6
Brewers' dried yeast	5.0	5.0
Glucose (cerelose)	25.0	25.0
Lard	3.9	
Corn oil		4.0
Ruffex ³	3.9	4.0
Minerals ⁴	4.0	5.0
Vitamins and antibiotic ⁵	+	+
Starch ⁶	36.9	36.4

¹ "Alpha protein" produced commercially by the Glidden Co., Chicago, Illinois.

² "Alpha protein" that had been washed with water as described in the text.

³ A commercial rice hull preparation by the Fisher Scientific Co.

⁴ The following ingredients were included in the mineral mixture for rations A and B, respectively: $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, 49.6% and 70.0%; CaCO_3 , 24.8% and 10.0%; NaCl , 24.8% and 20.0%; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.48% and 0.44%; KI , 0.03% and 0.03%; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.06% and 0.08%; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.03% and 0.03%; CoCl_2 , 0.015% and 0.013%; and ZnCl_2 , 0.015% and 0.013%.

⁵ The following vitamins were added to each 100 lb. of basal mixture: thiamine hydrochloride, 95 mg; riboflavin, 140 mg; niacin, 468 mg; calcium pantothenate, 670 mg; pyridoxine hydrochloride, 110 mg; folic acid, 27 mg; vitamin A, 100,000 I.U. and vitamin D, 10,000 I.U., both supplied by a concentrated fish oil; mixed tocopherols, 9 mg; and vitamin B_{12} , 1 mg. Aureomycin hydrochloride was added at the rate of 500 mg per 100 lb. In addition, each pig in trial A received 3.4 mg of mixed tocopherols orally twice during the experiment.

⁶ In the formation of experimental rations, DL-methionine, starch, and glycine were added to bring the total ingredients to 100%.

and differed from ration B in that ration B contained water-washed isolated soybean protein in place of the commercial isolated soybean protein, corn oil in place of lard, and a slightly different mineral mixture. Some difficulties were encountered in other studies subsequent to trial A which were believed to

be caused by sulfite present in the isolated soybean protein. The isolated soybean protein was washed with water by the following procedure. The dry commercial isolated soybean protein was autoclaved in shallow pans for 10 minutes at 10-lb. pressure. The autoclaved protein was then mixed in 30 to 35-lb. batches with approximately 15 gallons of tap water. The protein was allowed to settle, and the supernatant liquid was then siphoned off. Each batch of protein was washed with

TABLE 2
*Amino acid content of the basal rations*¹

	PER CENT OF RATION	
	A	B
Arginine	1.52	1.60
Histidine	0.51	0.52
Isoleucine	1.11	1.19
Leucine	1.64	1.81
Lysine	1.34	1.31
Methionine ²	0.26	0.25
Cystine ³	0.26	0.27
Phenylalanine	1.15	1.21
Tyrosine	0.70	0.63
Threonine	0.78	0.78
Tryptophan	0.22	0.22
Valine	1.32	1.44

¹ Determined by microbiological techniques to be published in detail later.

² Before supplementation with DL-methionine.

³ Cystine analysis for isolated soybean protein was determined by Dr. R. J. Block, New York Medical College, New York, N. Y.

three changes of water. After the third wash, the protein was pressed as free from water as possible in a screw press and was then dried in shallow trays in a drying room with a maximum temperature of 70°C. The washed, dried, and ground protein had the same physical appearance as the unwashed product. Miller ('51) found that the isolated soybean protein washed in ~~this manner~~ contained not more than 0.1% sulfite calculated as sodium sulfite as compared with approximately 1.1% in the unwashed protein.

The amino acid analyses of the two basal rations, shown in table 2, indicate that there was no significant change in the content of the essential amino acids caused by washing the protein. The only difference noted in the chemical composition of the unwashed and washed soybean protein was the ash content. The ash was reduced from 1.7% to 0.5% by the washing process.

In trial A, two levels of DL-methionine were added to the basal mixture to give three experimental rations of varying methionine content. A 4th lot of pigs was fed the basal ration plus 0.1% choline chloride.

In trial B, three levels of DL-methionine were added to the basal mixture to give 4 experimental rations of varying methionine content. Glycine was added to equalize the protein ($N \times 6.25$) content of each ration at 22%. A 35-day feeding period was used in trial A. The pigs in trial B were fed for only 28 days.

Growth rates, feed efficiency, and nitrogen balances were used as measures of the responses of weanling Yorkshire pigs to the different treatments. Daily feed intakes were equalized weekly at approximately 5% of the body weight. For nitrogen balances, fecal and urine collections were made over a 5-day period following a preliminary period of a minimum of 10 days.

RESULTS

The results of the two tests are given in tables 3 and 4. Since the initial weights of the lots in trial B differed considerably, the average daily gains are also presented as percentages of the initial weights. The data reported in table 3 clearly show that ration A was deficient in methionine. There was a 20% improvement in growth rate ($P \leq .02$) on 12% less feed ($P \leq .01$) when the ration was supplemented with 0.3% DL-methionine to make 0.56% total methionine. When the ration contained 0.36% methionine, neither the rate of gain nor the feed efficiency was as good as when the diet contained 0.56% methionine. The data for the apparently digested nitrogen retained in the body show that the increased growth of pigs re-

ceiving supplements of methionine involved storage in the form of protein tissue.

The results of trial B show that the basal ration plus 0.2% DL-methionine, containing a total of 0.45% methionine in the ration, was adequate for maximum growth, feed utilization

TABLE 3

Average data for pigs receiving various levels of DL-methionine in the isolated soybean protein ration A

	LOT NO. AND RATION			
	1 Basal	2 Basal + 0.1% methionine	3 Basal + 0.3% methionine	4 Basal + 0.1% choline chloride
Total methionine in ration (%)	0.26	0.36	0.56	0.26
Total methionine plus cystine in ration (%)	0.52	0.62	0.82	0.52
Number of pigs	4 ¹	6	6	6
Days on test	35	35	35	35
Initial weight (lb.)	35.0	33.6	34.1	33.9
Daily gain (lb.)	1.01	1.07	1.21 ²	1.03
Daily gain (% of initial weight)	2.88	3.18	3.55	3.04
Daily feed intake (% of body weight)	4.24	4.16	4.22	4.23
Feed per 100 lb. gain (lb.)	221	204 ³	193 ⁴	213
Apparent digestibility of nitrogen (%)	94	94	94	95
Apparently digested nitrogen retained (%)	43	50	52	44

¹ Two pigs were removed because of pneumonia.

² Significant over lots one and 4 at 2% level.

Significant over lot two at 5% level.

³ Significant over lot one at 2% level.

⁴ Significant over lots one and 4 at 1% level.

Significant over lot two at 5% level.

and nitrogen retention. The pigs grew 8% faster on 7% less feed when the basal ration was supplemented with 0.2% DL-methionine. The growth rates for the three methionine supplemented lots were similar when expressed as percentages of the initial weights. Also, there were no differences in feed efficiency for the three lots receiving added methionine. Al-



though the differences in rate of gain and feed efficiency between the unsupplemented basal lot and the lot that received the ration containing 0.45% methionine appear real, none of the differences in rate of gain or feed efficiency was statistically significant. The results in trial A indicate that a level of 0.36% methionine in the ration is not adequate for optimum growth; and in trial B, the ration was not improved by the addition of

TABLE 4

Average data for pigs receiving various levels of DL-methionine in the washed isolated soybean protein ration B

	LOT NO. AND RATION			
	1 Basal	2 Basal + 0.2% methionine	3 Basal + 0.4% methionine	4 Basal + 0.6% methionine
Total methionine in ration (%)	0.25	0.45	0.65	0.85
Total methionine plus cystine in ration (%)	0.52	0.72	0.92	1.12
Number of pigs	5	3 ¹	5	5
Days on test	28	28	28	28
Initial weight (lb.)	29.1	32.1	29.4	29.8
Daily gain (lb.)	0.77	0.92	0.85	0.84
Daily gain (% of initial weight)	2.65	2.85	2.87	2.82
Daily feed intake (% of body weight)	4.46	4.38	4.41	4.36
Feed per 100 lb. gain (lb.)	231	215	215	216
Apparent digestibility of nitrogen (%)	92	91	93	91
Apparently digested nitrogen retained (%)	36	42	36	39

¹ Two pigs died during the experiment. See text for details.

methionine beyond a level of 0.45% of the ration. From these data it appears that for the 22% protein ration used, the methionine requirement for growth of weanling pigs is approximately 0.45% of the ration, when the ration contains 0.27% cystine, or a total of 0.72% of methionine plus cystine.

In trial A, there was no significant improvement in growth or feed required per unit of gain when the basal ration was supplemented with 0.1% choline chloride. This indicates that

there was no deficiency of choline chloride or labile methyl groups in the basal ration. Analysis of the "alpha protein" and brewers' dried yeast showed that the ration contained 0.024% choline.

The average gains of the pigs in trial A were greater than the expected daily gain for pigs of that weight as given by the National Research Council ('50). The growth rates of pigs in trial B were slower than the growth rates of comparable lots of pigs in trial A. Two reasons are apparent for this difference. The pigs in trial A weighed more at the start of the test and were fed for 35 days in comparison to 28 days in trial B. Since the manner of feeding limited the individual daily feed intake to approximately 5% of the body weight, the larger pigs would be expected to gain at a faster rate on comparable rations. Likewise, with a longer feeding period, the pigs would gain more because of their greater size toward the end of the feeding period.

DISCUSSION

During the second week in trial B, one pig in lot two and one in lot 4 suddenly developed a flaccid type of paralysis. Both pigs recovered within 48 hours following an injection of 333 mg of thiamine hydrochloride.

One pig in lot two of trial B died quite suddenly on the 16th day of the experiment. He had been eating well and making excellent gains (0.91 lb. per day). Just prior to death, he was bloated and appeared to be in great pain. Autopsy showed no cause for death although bloat was evident. On the 25th day, another pig in lot two died quite suddenly. Autopsy disclosed no apparent cause for death although some evidence of bloat was observed. It is interesting to note that Bell et al. ('50) also reported that two pigs receiving a soybean oil meal-purified ration died suddenly. These pigs also showed bloat; however, in addition they were edematous in the inner thigh in the scrotum region and had meningeal hemorrhages. No final diagnosis was made.

Lyman and Elvehjem ('51) reported studies in which as much as 70% of the thiamine was destroyed in a purified ration

in 6 days. When it became evident that the rations in trial B might be deficient in thiamine, new rations were mixed. These rations were kept at a temperature of approximately -18°C . for the last 10 days of the experiment, and for one pig in each lot the refrigerated rations were fed for the last 24 days. In addition, a vitamin mixture equivalent to the amount already present in the ration was added to the evening feeding for each pig to insure against a possible vitamin deficiency. Both of the pigs that died were receiving the refrigerated ration for at least 7 days, and the extra vitamin supplement for at least 4 days before they died.

Inasmuch as it was impossible to attribute the cause of death to any of the rations fed, and since only two pigs out of 44 in two experiments were thus affected, the data obtained are believed to be valid. It is unfortunate that the data for the lot receiving 0.45% methionine, the fastest growing group, in trial B were for only three pigs; however, it is believed that the conclusions are justified, even with this limitation.

As the methionine plus cystine requirement of growing-fattening pigs is shown to be not more than 0.69% of the 22% protein ration containing soybean oil meal (Curtin et al., '52) and approximately 0.72% of the 22% protein ration containing isolated soybean protein, it is suggested that the requirement is approximately 0.7% of the ration containing 22% protein or 3.2% of the protein. Calculation shows the corresponding value reported by the Purdue workers (Shelton et al., '51) to be 2.86% of the protein which is 10% lower than the requirement as reported herein. The Purdue workers used a purified diet that required supplementation with 0.5% of a combination of DL-methionine and L-cystine, or DL-methionine alone for optimum growth. The crystalline amino acids might be expected to be more highly digestible than the amino acids present in natural feeds such as the soybean protein used in this study. Only 0.2% DL-methionine added to the isolated soybean protein-brewers' dried yeast ration was necessary for optimum performance in the studies reported herein. The slightly lower digestibility of methionine and cystine present in natural feeds,

or a difference in the pigs used, might account for the difference in the requirement found in this study and that reported by the Purdue workers.

The isolated soybean protein rations contained 0.26% and 0.27% cystine. Earlier work (Curtin et al., '52) has indicated that 0.38% cystine probably can replace a corresponding amount of methionine. Previously, Shelton et al. ('51) found that L-cystine could replace at least 50% of the methionine requirement of the pig.

SUMMARY

Two experiments were conducted to study the methionine requirement of weanling pigs fed a ration containing 22% protein from isolated soybean protein and brewers' dried yeast. From the data obtained, the methionine plus cystine requirement for weanling pigs appears to be approximately 0.7% of the ration containing 22% protein. This corresponds to 3.2% of the protein. Cystine apparently can be used to replace methionine in the ration up to at least 1.7% of the protein.

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THE RELATION BETWEEN VITAMIN B₁₂
DEFICIENCY AND LACTATION
IN RATS FED PURIFIED
CASEIN RATIONS

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ONE FIGURE

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It has been reported from this laboratory and elsewhere that the weaning weights of young rats nursed by mothers on vitamin B₁₂-deficient rations were suboptimal (Zucker et al., '48; Dryden, Hartman and Cary, '49; Emerson, Kummer and Zanetti, '49; Lepkovsky et al., '50, '51; Nell and Phillips, '50; Watts et al., '50) and that their post-weaning weight gains were likewise less than when the young had been nursed by mothers on rations containing vitamin B₁₂ or B₁₂-active materials (Cary and Hartman, '43-'47; Hartman et al., '49; Emerson et al., '50). Lepkovsky et al. ('51) reported that the impaired lactation observed with first litter young of mothers transferred at positive mating to a vegetable protein B₁₂-deficient diet was apparently the result of an inadequate amount of milk rather than a milk of poor quality, but they attributed this condition to unknown factors inherent in their vitamin B₁₂-deficient ration rather than to a vitamin B₁₂ deficiency as the major cause.

In this paper the feeding of certain vitamin B₁₂-deficient casein-containing rations to the mother is considered in rela-

¹ Formerly Head, Division of Nutrition and Physiology; retired Feb., 1950.

tion to its effect upon the young during the pre-weaning and post-weaning period, and consideration is given to the manner in which B₁₂ deficiency affects lactation. Survival during the pre-weaning period of young born to mothers on these rations has been discussed in a previous paper (Dryden, Hartman and Cary, '52).

TABLE 1
Composition of rations¹

	RATION NUMBER							
	47	192	194	260	262	300	302	443
	%	%	%	%	%	%	%	%
Dextrin ²	45.50	54.85	45.28	30.50	40.38	40.32	54.32	53.43
Lactose (U.S.P. XIII)	15.00	15.00	15.00	..	1.50
Casein (10x hot alcohol extd.) ³	20.00	30.00	20.00	20.00	20.00	30.00	30.00	30.00
DL-methionine	..	0.20	0.20	0.20
Yeast, dried brewers' ⁴	20.00	..	20.00	20.00	10.00
Salts (Hawk and Oser, '31)	4.50	4.50	4.50	4.50	4.50	4.50	4.50	4.50
Succinylsulfathiazole
Cottonseed oil	9.85	9.85	9.85	9.85	9.85	9.85	9.85	9.85
Fish liver oil ⁵	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vitamins added	..	0.46	0.03	..	0.12	0.18	0.18	0.37
Vitamins: mg/100 gm ration								
Thiamin hydrochloride	..	1.6	0.5	0.8	0.8	1.6
Riboflavin	..	1.6	0.5	0.8	0.8	1.6
Pyridoxine hydrochloride	..	1.6	0.5	0.8	0.8	1.6
Calcium pantothenate	..	20.0	3.1	5.0	5.0	10.0
Choline chloride	..	240.0	75.0	120.0	120.0	240.0
Nicotinic acid	..	20.0	3.1	5.0	5.0	10.0
Inositol	..	20.0	3.1	5.0	5.0	10.0
Para-aminobenzoic acid	..	120.0	18.8	30.0	30.0	60.0
Biotin	..	0.01	0.006	0.01	0.01	0.02
Pteroylglutamic acid ⁶	..	0.20	0.06	0.10	0.10	0.20
Ascorbic acid	..	10.0	10.0	..	3.08	4.86	4.86	10.0
Alpha-tocopherol acetate	..	20.0	20.0	..	6.25	9.72	9.72	20.0
2-methyl, 1, 4-naphthoquinone	..	0.5	0.5	..	0.16	0.25	0.25	0.5

¹ Ration 45 was the same as ration 47, and ration 197 was the same as ration 194 except that in each case 10% dextrin replaced 10% of yeast. Rations 195, 301 and 303 were the same as rations 192, 300 and 302, respectively, except that in each case 5% dextrin replaced 5% casein.

² "Amidex," a dextrinized cornstarch manufactured by the Corn Products Refining Co., New York, N. Y.

³ For preparation of casein, see Hartman, Dryden and Cary ('51).

⁴ Strain G, Anheuser-Busch Co., St. Louis, Mo.

⁵ "Navitol with Viosterol (concentrated oleovitamin A and D) U.S.P.," E. R. Squibb and Sons, New York, N. Y. According to manufacturer, contained per gram 65,000 I.U. of vitamin A and 13,000 I.U. of vitamin D.

⁶ Kindly supplied by Dr. E. L. R. Stokstad and Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

EXPERIMENTAL PROCEDURE

The composition of the B₁₂-deficient rations used in these experiments is described in table 1; all of them are essentially modifications of ration 47 as indicated in the table. All of the rations and the distilled water were supplied ad libitum. Except as otherwise indicated, supplements of 15 units/ml APA liver extract² or crystalline vitamin B₁₂³ were administered orally, either daily or three times per week.

The mothers used in these experiments were of 4 types: (1) those fed the stock colony ration⁴; (2) those transferred at parturition or a day or so thereafter from the stock colony ration and maintained on the experimental ration during lactation; (3) those continued on the experimental ration beyond the initial lactation described in 2; and (4) rats whose ancestors had been maintained on the experimental ration for one or more generations. During lactation, the mothers and their young were bedded on wood shavings; beyond 28 days of age, the young were kept in individual cages on raised screen floors.

Litters born while the mothers were on the stock ration and litters in the "generation" experiment shown in table 4 were reduced to 6 in number at birth; all of the others were reduced to 6 at the end of one week. The young that were retained were weaned at 25 days of age.

RESULTS AND DISCUSSION

Pre-weaning weights of young

The effect of vitamin B₁₂ deficiency upon the weights of young rats nursed by mothers on alcohol-extracted casein rations is shown in table 2 for various ages during the lactation period. It can be seen that, although differences in body

² Lederle.

³ The crystalline vitamin B₁₂ was kindly supplied by Dr. D. F. Green and Dr. M. A. Schooley, Merck and Co., Inc., Rahway, N. J.

⁴ Composition of stock ration: yellow corn meal 69.5; linseed oil meal 14.0; meat scrap 9.0; casein (commercial) 4.0; alfalfa meal 2.0; bone meal 2.0; sodium chloride 0.5. Supplements of lettuce and carrots were fed once a week.

weight of the young occurred between B_{12} -deficient and B_{12} -supplemented rations in all cases before weaning, there was considerable variation in the time when a significant difference⁵ could first be observed. Thus, of the young born to mothers transferred at parturition from the stock ration to the experimental rations (groups 1, 3, 5, 7, 9), significant differences in weight as early as 14 days occurred between rats on the B_{12} -deficient ration and those on the B_{12} -supplemented ration in only two of the 5 groups—with the male young on the lactose-containing yeast-free ration (300) and with both male and female young on one of the yeast rations (194). By 21 days of age, however, with the exception of one small group, significant differences in weight were observed with all groups of animals on all rations except 192. It has been shown in a previous publication (Hartman et al., '51) that feeding ration 192 was often conducive to the intestinal synthesis of vitamin B_{12} -active material but even here, significant differences in weight between the young from B_{12} -supplemented mothers and those from B_{12} -deficient mothers could be observed by the 25th day of age.

The percentage increase in weight of the young from the B_{12} -supplemented mothers above that of the young from the B_{12} -deficient mothers also varied in a similar manner from ration to ration at the various ages. Thus at 25 days of age, it ranged from 5% to 36%, being highest on ration 300 and lowest on ration 192.

Because of the poor survival during the first few days after birth of the offspring of mothers maintained on the yeast-free B_{12} -deficient rations through additional gestations and lactations beyond the initial nursing period, comparisons of pre-weaning weights between the young from B_{12} -deficient and those from B_{12} -supplemented mothers (groups 2, 4, 6, 8)

⁵ The symbol ** adjacent to or in connection with a *t* or *F* value indicates statistical significance at or less than the 1% level; * indicates significance at the 5% level or between the 5% and 1% levels; no * indicates no statistically significant difference.

are limited in some cases to very small numbers. It would appear, however, that in most cases during these later lactations, significant differences occurred at an earlier age and were greater in magnitude than they had been during the initial lactation on the experimental rations. Thus the body weights at 25 days of age of the young from B₁₂-supplemented mothers were 14 to 55% greater than those of the young from B₁₂-deficient mothers, with the greatest differences occurring on ration 300 and the smallest on ration 192 as before.

Change in weight of the mother during lactation

The change in weight of the mother during the nursing period is also shown in table 2. There was no significant difference in any group between the changes in weight of the B₁₂-deficient and B₁₂-supplemented mothers. After the mothers were maintained on the experimental rations through successive gestation and lactation periods, there was a tendency for both unsupplemented and supplemented mothers to lose more weight than they did during the lactation immediately following the transfer to the experimental ration; this tendency was highly significant ($t=5.6^{**}$) with the supplemented mothers.

Post-weaning weight gains

The effect of feeding a supplement of vitamin B₁₂ to mothers placed on a B₁₂-deficient ration is reflected not only in the weights of the young preceding weaning but also in their post-weaning weight gains. A comparison of such weight gains is shown in table 3 between the young whose mothers were put on a B₁₂-deficient ration at parturition and those whose mothers were fed either the stock ration or a B₁₂-supplemented ration during lactation. (Similar effects were obtained whether the mothers were continued on the stock ration or put on the deficient ration supplemented with either APA liver extract or crystalline vitamin B₁₂.)

TABLE 2
Effect of vitamin B₁₂ deficiency upon body weights of young and changes in weight of mother during lactation

GROUP	RATION OF MOTHER DURING GESTATION	SUPPLEMENT ¹ FED TO MOTHER	NO. OF MOTHERS	NO. OF YOUNG AT 25 DAYS OF AGE ²		BODY WEIGHT OF YOUNG AT:												AVERAGE CHANGE IN WT. OF MOTHERS DURING LACTATION
				Males	Females	7 days of age ³		14 days of age		21 days of age		25 days of age						
						gm	t	gm	t	gm	t	gm	t					
														Males	Females	Males	Females	
1	Stock	None	13	47	29	16	29	1.3	30	0.0	46	1.2	58	2.2*	-2.6	0.6		
	Stock	B ₁₂	17	50	45	16	30		30		46		61		-5.5			
2	195	None	5	14	13	12	25	4.0**	26	2.3*	40	3.8**	40	2.9**	50	3.9**	-17.2	0.2
	195	B ₁₂	13	55	48	15	30		29		47		44		57		-16.1	
3	Stock	None	5	26	3	13	23	2.5*	23	1.6	34	6.0**	33	6.2**	42	6.8**	-3.4	1.2
	Stock	B ₁₂	5	19	10	14	25		26		41		42		59		+8.4	
4	301	None	1	3	3	12	22	5.6**	24	7.2**	33	8.7**	37	10.1**	44	9.2**	-42.0	
	301	B ₁₂	3	22	17	17	32		31		51		50		68		-17.7	
5	Stock	None	5	25	5	13	24	0.8	24	0.7	37	2.5*	37	1.1	48	5.1**	-3.0	1.0
	Stock	B ₁₂	4	18	6	13	25		25		40		40		55		-8.2	
6	303	None	1	2	2	16	28	0.1	27	1.5	38	1.0	38	0.3	43	2.9**	-1.0	
	303	B ₁₂	4	20	18	12	27		25		42		39		55		-20.6	
7	Stock	None	10	39	19	15	25	4.4**	24	4.1**	39	5.6**	38	4.0**	51	10.4**	-3.1	2.1
	Stock	B ₁₂	10	50	10	14	27		27		43		44		60		+7.3	
8	197	None	7	38	24	14	25	5.6**	23	5.7**	38	6.4**	35	5.3**	49	6.4**	-1.2	1.2
	197	B ₁₂	9	38	38	15	30		29		48		46		62		-7.1	
9	Stock	None	10	39	17	17	31	1.4	29	0.7	46	3.0**	41	2.4*	59	3.8**	-0.8	1.3
	Stock	Liv. ext.	10	38	17	16	32		30		51		47		66		+11.8	

¹ Crystalline vitamin B₁₂—1 or 2 µg/day; 15 units/ml APA liver extract—0.10 ml/day.

² Some groups contained slightly larger numbers at earlier ages.

³ At 7 days of age, males and females were not weighed separately.

When the mothers were fed a B₁₂-containing ration, the 4-week gains in weight of the young subsequent to 28 days of age were 41 to 57% greater for the males, and 30 to 31% greater for the females when the young were fed a source of

TABLE 3
Effect of maternal ration during lactation upon post-weaning weight gains of young

GROUP	RATION OF MOTHER DURING LACTATION ¹	SUPPL. TO MOTHER'S RATION ²	RATION OF YOUNG AFTER 28 DAYS OF AGE	NO. OF LITTER-MATE PAIRS OF YOUNG	4-WEEK POST-WEANING WEIGHT GAINS ³				
					No supplement		Vitamin B ₁₂ ⁴		<i>t</i>
					<i>gm</i>	<i>t</i>	<i>gm</i>	<i>t</i>	
1 (males)	47	None	45	349	88		154		44.4**
	Stock	None	45	18	114	6.8**	164	2.3*	9.2**
	Stock	None	Stock	10	157		...		
2 (females)	47	None	45	81	57		103		22.8**
	Stock	None	45	14	87	8.8**	114	3.6**	6.6**
	Stock	None	Stock	15 ⁵	98		...		
3 (males)	47	None	45	26	85		160 ⁶		12.9**
	47	Liv. ext.	45	26	108	3.7**	161 ⁶	0.1	6.1**
4 (females)	47	None	45	11	56		104 ⁸		6.9**
	47	Liv. ext.	45	11	76 ⁷	4.6**	99 ⁸	0.7	5.0**
5 (males)	302	None	303	5	68		126		6.4**
	302	B ₁₂	303	4	96	2.3	151	3.3*	4.3*
6 (males)	300	None	301	5	53		146		8.1**
	300	B ₁₂	301	4	93	3.2*	131	1.2	2.8

¹ Fed to young until about 28 days of age.

² One-tenth ml/day, 15 units/ml APA liver extract or 1 µg/day crystalline B₁₂ as indicated.

³ Twenty-eight to 56 days of age.

⁴ Liver extract: 0.02–0.10 ml/day (group 1); 0.02–0.05 ml/day (group 2); 0.02 ml/day (groups 3, 4). Crystalline B₁₂: 2 µg/day (groups 5, 6).

⁵ Weaned at 21 days of age.

⁶ Twelve rats.

⁷ Ten rats.

⁸ Six rats.

B₁₂ than when they were given the B₁₂-deficient ration alone. On the other hand, when the mothers were maintained on an unsupplemented B₁₂-deficient ration during the nursing period, the 4-week post-weaning weight gains of the young fed a sup-

plement of B_{12} -active material were for two of the rations (45, 303) 75 to 88% greater, and for the other ration (301) 175% greater than those of the young fed the B_{12} -deficient ration alone.

The smaller differences between the young fed B_{12} -deficient and B_{12} -supplemented rations which were obtained when the mothers were fed a B_{12} -active ration were apparently due primarily to increased weight gains of the B_{12} -deficient young. Feeding B_{12} to the mother apparently had much less effect on the 4-week post-weaning weight gains of the young when the latter also received a source of B_{12} . Under these conditions, only three of the 6 groups showed a significant difference, the young from mothers who had received B_{12} having higher weight gains in these instances. Two of the groups in which no significant differences were observed were the only groups in which the comparisons were made directly between litter mates.

B_{12} -deficient rations over several generations

The 28-day weights and the subsequent 4-week post-weaning weight gains of the young maintained on yeast-containing B_{12} -deficient rations over several generations are shown in table 4. The parent generation mothers were transferred from the stock ration to the experimental ration at parturition of a litter, and carried through several matings on this ration. Their young born on the deficient ration were divided into two groups at 28 days of age, one maintained on a similar B_{12} -deficient ration and the other (litter mates of the first group) on the same ration plus a supplement of liver extract. These two groups were carried through the F_2 and F_3 generations.

The 28-day weight differences between the young from supplemented and unsupplemented mothers were much the same for both sexes in all generations (21 to 26% greater for the groups from supplemented mothers) except in the

TABLE 4

Changes in rate of growth of young as a result of keeping mothers on vitamin B₁₂-deficient yeast-containing rations over several generations

GENERAL- TION OF MOTHERS	RATION OF MOTHERS DURING LACTATION		MALE YOUNG				FEMALE YOUNG				CHANGE IN WT. OF MOTHERS DURING LACTATION	
	No. ¹	Suppl.	28-day wt.	4-week post-weaning weight gains		28-day wt.	4-week post-weaning weight gains		28-day wt.	4-week post-weaning weight gains		
				No. suppl.	Liv. ext. ²		No. suppl.	Liv. ext. ²		No. suppl.		Liv. ext. ²
Parent ³	47	None	63 ± 2.1 ⁴ (29) ⁵	79 ± 3.0 (17)	155 ± 5.6 (12)	61 ± 2.0 (29)	61 ± 1.8 (16)	93 ± 1.9 (13)	61 ± 1.8 (16)	93 ± 1.9 (13)	-5 ± 1.6 (12) ⁶	
F ₁	47	None	57 ± 0.9 (75)	80 ± 2.6 (31)	129 ± 3.2 (24)	54 ± 0.8 (77)	54 ± 2.8 (42)	92 ± 2.8 (20)	54 ± 2.8 (42)	92 ± 2.8 (20)	-13 ± 2.8 (31)	
F ₁	47	Liv. ext. ²	70 ± 1.0 (85)	122 ± 3.5 (42)	139 ± 3.9 (32)	66 ± 0.7 (92)	79 ± 1.8 (43)	93 ± 1.9 (38)	79 ± 1.8 (43)	93 ± 1.9 (38)	-15 ± 2.4 (34)	
F ₂	47	None	44 ± 2.1 (33)	67 ± 4.1 (14)	121 ± 4.4 (9)	40 ± 2.2 (23)	52 ± 2.5 (16)	89 ± 2.8 (5)	52 ± 2.5 (16)	89 ± 2.8 (5)	+1 ± 4.2 (10)	
(to 2/1/47)	47	Liv. ext. ²	72 ± 1.8 (14)	124 ± 6.7 (8)	149 ± 6.7 (6)	64 ± 1.3 (16)	80 ± 3.1 (8)	86 ± 5.7 (7)	80 ± 3.1 (8)	86 ± 5.7 (7)	-17 ± 5.3 (7)	
F ₂	47	None	62 ± 1.0 (155)	96 ± 3.8 (41)	138 ± 2.9 (32)	56 ± 0.9 (106)	63 ± 2.0 (31)	89 ± 2.4 (18)	63 ± 2.0 (31)	89 ± 2.4 (18)	-9 ± 1.9 (51)	
(2/1/47 and later)	47	Liv. ext. ²	75 ± 1.5 (69)	132 ± 4.7 (16)	142 ± 6.1 (13)	68 ± 1.5 (53)	86 ± 2.2 (14)	93 ± 3.6 (9)	86 ± 2.2 (14)	93 ± 3.6 (9)	-19 ± 3.7 (24)	
F ₃	47	None	62 ± 1.2 (79)	96 ± 11.3 (8)	148 ± 6.0 (8)	58 ± 1.0 (95)	69 ± 3.3 (8)	96 ± 5.9 (6)	69 ± 3.3 (8)	96 ± 5.9 (6)	-11 ± 3.1 (41)	
F ₃	47	Liv. ext. ²	77 ± 1.1 (42)	73 ± 1.3 (19)	-17 ± 3.9 (11)	

¹ Also fed to young until 28 days of age or thereafter; young received ration 45 from that time on.

² Five-hundredths ml/day, 15 units/ml APA liver extract.

³ Figures include only young born while mothers were on experimental rations.

⁴ Standard error of the mean.

⁵ Figures in parentheses indicate the number of young.

⁶ Figures in parentheses indicate the number of litters.

early stage of the F_2 generation, where the average weight was 64% greater for the males and 60% greater for the females nursed by supplemented mothers.

If the 4-week post-weaning weight gains of the young from B_{12} -deficient mothers are considered, it can be seen that there was a significant tendency for those of both the unsupplemented and B_{12} -fed young to drop to a low point during the early stage of the F_2 generation, and after that for the B_{12} -fed young to regain the position held at the beginning of the experiment, and for the unsupplemented young to attain weight gains considerably in excess of those at the beginning of the experiment. This tendency is not pronounced in the young from the B_{12} -fed mothers.

It will be noted again that the unsupplemented young from mothers fed liver extract gained considerably more weight in the post-weaning period than did the young from mothers not fed liver extract. It is also evident that in these later generations, if the young were fed liver extract, the feeding of liver extract to the mothers had no effect on the post-weaning gains of the female young, but did have a significant effect in the case of the male young in the F_1 and early F_2 generations.

That the decrease in weight gains in some groups during the early part of the experiment might represent a gradual depletion of some factor not found in liver extract seems quite doubtful since the average weight gains of the supplemented young that came from mothers receiving liver extract were not significantly lower in the F_2 generation than in the parent generation.

The differences in gains in weight between the unsupplemented young from unsupplemented mothers and the supplemented young from supplemented mothers for the early part of the experiment were not markedly different from those found for the young from mothers put on the deficient rations at parturition (table 3, groups 3 and 4); however, the differences

in the latter part of the experiment were smaller. To a large extent these smaller differences were due to the greater growth of the unsupplemented young from the unsupplemented mothers. Thus, of such young in the late F₂ and the F₃ generations, 52% grew more than 100 gm, and 18.5% grew more than 120 gm while none of their ancestors born to the parent generation mothers grew over 100 gm for the 4-week period. There were, moreover, in this later period some growths (such as 146 gm and 162 gm) that were as high as the average for the young receiving the liver extract; such growths would seem to represent intestinal synthesis of B₁₂-active material, something that apparently did not occur in the earlier generations. It should be noted, however, that, considering all of the young in our colony that were reared by mothers transferred from the stock ration to B₁₂-deficient ration 47 in the previous two years, 21% of those growing during the same period of the year as the young belonging to the latter part of the F₂ generation and the F₃ generation in this experiment gained 120 gm or more during the 4-week post-weaning period.

It would appear then that retaining animals over several generations on these B₁₂-deficient yeast-containing casein rations did not produce effects on lactation that were very much more pronounced than those that were observed either in the lactations in the parent generation, if comparative weights during the lactation period are considered, or even in the lactation immediately subsequent to placing the mothers on the deficient ration at parturition, if the criterion used is the subsequent post-weaning weight gains.

Storage of vitamin B₁₂ in the young

The effect of placing mothers on a B₁₂-deficient ration at parturition is shown not only in the pre-weaning weights and post-weaning weight gains but also in the storage of vitamin B₁₂ in the bodies of the young. Lewis, Register and Elvehjem ('49) showed that, of the organs and tissues tested, the site of the greatest B₁₂ concentration in the body of the rat was the

kidney and that the liver also contained appreciable B_{12} when this vitamin was included in the diet. Richardson et al. ('51) showed that the amount of B_{12} in the livers of 28-day-old rats was increased when a source of B_{12} had been included in the diet of the mother, but, since the diet presumably had also been available to the young, it is not clear from the data presented whether the increase occurred through a change in the B_{12} intake from the milk of the mother or through an increased intake from the diet by the young themselves. It seemed desirable therefore to run a similar test on rats not old enough to be taking in solid food.

For this experiment, test organs were obtained from young rats at 13 to 15 days of age. These young came from stock mothers who had been placed at parturition on ration 260 either with or without an oral supplement of crystalline B_{12} ($2\text{ }\mu\text{g/day}$); no significant differences in the weight of the test young or in the weights of their kidneys or livers were observed at the time they were sacrificed. The organs were assayed by a rat-growth assay method involving the use of 38- to 45-day-old B_{12} -deficient rats fed ration 262 and reared by mothers fed ration 260 during lactation. Each animal receiving test organs was given either the liver or both kidneys of one young rat per day for 12 days during the 14-day test period.

The assay indicated that the kidneys of the young from the mothers fed the B_{12} -deficient ration alone contained only about 56% ($t=1.5$) as great a concentration of B_{12} as did the kidneys of the young from the mothers receiving a B_{12} supplement, while the livers contained only about 26% as much ($t=2.9^*$). (This more rapid depletion of the liver is in agreement with the findings of Lewis et al., '49.) These differences occurred at a time when there could be no possibility of the young having consumed food other than their mother's milk.

Influence of access of young to feces of mother

Since the young from mothers fed a supplement of vitamin B_{12} in addition to the B_{12} -deficient rations grew better than

those from the mothers on the B₁₂-deficient rations alone, it was evident that the young themselves must have been receiving a supply of B₁₂ when it was fed to the mothers. The experimental technique used in feeding the supplement to the mothers, that is, by means of a hypodermic syringe with the blunted needle placed well back in the throat or, more frequently, directly into the esophagus itself, would seem to preclude the possibility of the young securing any of the B₁₂ from about the mouth and lips of the mother. It is not clear, however, that the B₁₂ was necessarily secured by the young through the milk, since it would appear possible that some might be obtained from the mother's excreta.

Chow et al. ('51), Barbee and Johnson ('51) and Yamamoto et al. ('51) showed that when B₁₂ was administered orally to rats, excretion of the vitamin occurred primarily through the feces. It has been observed in this laboratory that B₁₂-active material is contained in the feces of rats receiving oral supplements of the vitamin, even at suboptimal doses, but not in the feces of rats fed such unsupplemented B₁₂-deficient rations as 47 and 262. While it would not seem that consumption of the excreta could explain weight differences occurring before the young were consuming solid food, yet such consumption would present a reasonable explanation where differences did not occur until 21 days of age or later.

The rats whose pre-weaning weights are shown in table 2 (ration 47) and whose post-weaning weight gains are shown in table 3 (groups 3 and 4) were used to test this possibility. In this experiment, mother rats were used in pairs, both members of a pair having littered on the same day or not more than one day apart. The litters of each pair were interchanged between the two mothers so that each mother of the pair nursed the same number of young (5 or 6), and so that all of the young reared by one mother were composed of the same number of siblings of the same sex as those reared by the other mother of the pair. Ten pairs of mothers were treated in this way. One mother of each pair received a supplement of liver extract (0.10 ml/day) throughout the nursing

period while the other mother received no supplement. It will be noted that differences in the average body weights of the young nursed to these two groups of mothers first became significant at 21 days of age and that during the 28th to 56th day of life, the male young from the supplemented mothers,

TABLE 5

The influence of access to feces from rats fed liver extract upon the comparative weight gains of young nursed to mothers fed a B₁₂-deficient ration either with or without a supplement of B₁₂-active material

AGE	AVERAGE WEIGHT DIFFERENCES BETWEEN YOUNG NURSED TO MOTHERS FED RATION 45 ALONE AND THOSE FROM MOTHERS FED RATION 45 + LIVER EXTRACT ¹				
	Males		<i>t</i>	Females	
	Cages not interchanged	Cages interchanged		Cages not interchanged	Cages interchanged
<i>days</i>	<i>gm</i>	<i>gm</i>		<i>gm</i>	<i>gm</i>
Body weights					
	(18 pairs) ²	(21 pairs)		(4 pairs)	(13 pairs)
0-1	— 0.1	— 0.1	..	— 0.2	— 0.2
7	— 0.8	0.9	..	— 3.3	— 0.1
14	0.2	2.5	..	— 0.7	1.6
21	3.6	5.2	..	3.3	5.9
25	6.4	7.0	..	5.9	9.2
28	10.0	9.3	0.2	9.2	13.4
Weight gains from 28 days of age					
	(13 pairs)	(13 pairs)		(2 pairs)	(9 pairs)
35	8.1	3.1	1.3	7.5	9.3
42	17.1	10.9	0.9	4.5	14.2
49	22.5	16.9	0.6	13.0	15.9
56	25.2	19.4	0.5	25.0	18.8

¹ Differences are designated positive where weights or weight gains of young nursed to mothers receiving liver extract are higher, and negative where lower.

² Only 17 young in liver extract group.

although receiving no supplement themselves, grew 27% faster, and the female young 36% faster than the corresponding young from the unsupplemented mothers.

In order to determine whether this extra growth could be due in some measure to B₁₂ obtained from the excreta of the liver-fed mothers, the two mothers of a pair and the young nursing to them, had, in some pairs, their cages interchanged

daily beginning at different periods during the nursing period and continuing to the 28th day of age, so as to give the young with each mother and the mothers themselves as nearly equal access as possible to the excreta of the mother receiving the liver extract.⁶

The data as presented in tables 2 and 3 represented the combined data of those litters whose cages were and were not alternated. In table 5 the pairs of litters which had their cages interchanged are compared with those which did not have their cages interchanged. It can be seen that at no time during the experiment — either during lactation or during the succeeding 4-week period — were the differences in weights or weight gains between the young nursed by supplemented mothers and those nursed by unsupplemented mothers significantly greater when the cages were not alternated than when they were alternated. It seems then that access to the excreta of the mother was not a significant factor in explaining the differences between the weight gains of the young from B₁₂-supplemented mothers and those from B₁₂-deficient mothers under our experimental conditions.

Quantity and quality of milk produced during lactation

Since it appears that young rats nursed to mothers fed a separate supplement of B₁₂-active material did not obtain a significant amount of B₁₂ either from the ration or supplement fed the mothers or from the mother's excreta, it would appear that they must have obtained B₁₂ through the mother's milk. This B₁₂ could have been obtained through a larger amount of milk containing the same concentration of B₁₂ as that given by the B₁₂-deficient mothers, through the same amount of milk containing a larger B₁₂ concentration or through a larger supply containing an increased concentration.

It seemed that the injection of B₁₂ into some of the young of a litter rather than feeding it to the mothers might throw

⁶ The shavings upon which the mother and young were nested, together with the excreta present, were replaced by clean shavings at about weekly intervals during the 28-day period.

some light on this question, since if injected young grew no better than uninjected young nursed to the same B_{12} -deficient mother, it would appear that the quantity of the milk was being affected by feeding B_{12} to the mothers. The results of such an experiment are shown in figure 1. No differences in body weight could be observed between injected and non-injected young before they were eating solid food, and no sig-

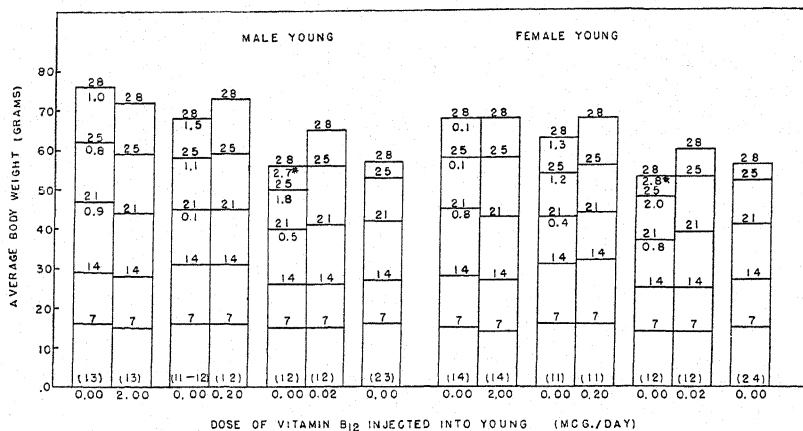


Fig. 1 Effect on pre-weaning weights of vitamin B_{12} injected into young nursed to mothers on B_{12} -deficient rations. Figures above lines indicate age of young (in days); figures below lines indicate t values for difference in weight between non-injected and injected young. Figures in parentheses at the bottom of each column indicate the number of young in each group. The young were derived from 8 or 9 mothers at each dose level. The doses shown are average daily doses; injections were actually made on the 4th, 8th, 12th, 16th, 20th and 22nd days of age.

nificant differences were noted until 28 days of age. Even then, a significant difference was obtained only when a sub-optimal dose was injected.

At the two higher doses, it appeared that some B_{12} was being transferred from the injected young to the non-injected young. Chow et al. ('50) and Barbee and Johnson ('51) showed that when B_{12} was injected into rats, excretion of the vitamin occurred primarily through the urine. In the present experiment, since it appeared that no B_{12} could have been secured

by the mother or uninjected young from the injection site, it would seem probable that the mother obtained some B₁₂ in the process of cleaning the young and then transferred some of it through her milk to the uninjected young. The results indicate that this was not a significant factor at the suboptimal dose injected.

The results of this experiment are in accord with the idea that the feeding of B₁₂ to the mother affected the quantity rather than the quality of milk produced by the mothers.

Since it would appear that the change in weight of the young after a short period of nursing would reflect primarily the amount of milk secured by the young from the mother, this indirect method was used in an effort to obtain some measure of the relative output of milk by B₁₂-deficient and B₁₂-supplemented mothers. Stock colony mothers were taken at parturition in pairs, both members of the pair having given birth to litters on the same day or not more than one day apart. They were placed on a B₁₂-deficient ration (260), one member of the pair receiving an oral supplement of crystalline B₁₂ (2 µg/day) and the other receiving no supplement. At 11 to 13 days of age, the young of both members of the pair were separated from their mothers in the late afternoon. The next morning the young were returned to the mothers, three of the young from the supplemented mother and three from the unsupplemented mother being given to each member of the pair to nurse. The weight changes of the young over the next 5 hours were then determined. The results are shown in table 6.

It appeared that the supplemented mothers were producing at this time about 20% more milk on the average than the unsupplemented mothers. Analysis of variance indicated that the difference in intake by the young occurred primarily between the two groups of the young previously reared by the supplemented mothers ("positive" young); only a small and non-significant difference occurred between the groups that were previously reared by the non-supplemented mothers ("negative" young). However, the difference between the "positive" young and the "negative" young was significant

only when both groups were nursed to supplemented mothers. Interpretation of the data is complicated somewhat since in each case a comparison must be made between the mother's own young and the foster young nursed by her; however, a similar experiment involving only supplemented mothers showed no significant difference between the amount of milk secured by a mother's own young and the foster young nursed

TABLE 6

*Effect of vitamin B₁₂ deficiency during lactation on quantity of milk produced*¹

	AVERAGE 5-HOUR WT. CHANGE PER NURSING YOUNG			Analysis of variance <i>F</i> young
	"Negative" young ²	"Positive" young ²	All young	
	gm	gm	gm	
Mothers fed no supplement	1.22	1.12	1.17	Not significant
Mothers fed vit. B ₁₂	1.28	1.51	1.40	Significant*
Analysis of variance: <i>F</i> mothers	Not significant	Significant**	Significant**	

¹ Seven pairs of mothers and 84 young were used in this experiment. At the time of the test, the young were 12 to 14 days of age and their average body weights were 25.4 gm for the young from the unsupplemented mothers and 25.6 gm for the young from the supplemented mothers. An 8th pair of mothers has been omitted since one mother of the pair did not nurse the young to any appreciable extent when they were given to her.

² "Negative" young are those previously reared by unsupplemented mothers; "positive" young are those previously reared by supplemented mothers.

to her when both mothers had received the same amount of vitamin B₁₂. Thus it would appear not only that the B₁₂-deficient mothers produced less milk but that there was less inclination for their young to take in additional milk when it was available.

While the evidence indicates that the B₁₂-supplemented mothers produced more milk than the B₁₂-deficient mothers, this does not necessarily mean that the milk produced by the

supplemented mothers did not also contain a greater concentration of B₁₂. Meyer et al. ('51) did not find a significant increase in the B₁₂ concentration (determined microbiologically) of rat milk when mother rats on purified casein rations were supplemented with 0.1 µg B₁₂ per day; however, they did obtain such an increase when the rats were fed an all-plant ration supplemented with higher levels of B₁₂.

Evidence obtained in this laboratory has shown that with such B₁₂-deficient rations as 262 and 47, the weight differences between supplemented and unsupplemented young during the post-weaning period are accompanied by a decreased food consumption by the unsupplemented young; thus when the supplemented young were limited to the food intake of the unsupplemented young, no differences in weight gain were observed. It would seem that if the same situation prevails during lactation, differences in the quality of milk would not be reflected by differences in the weight of the young unless the quantity of milk was also affected. It thus appears that differences in body weights that occurred before the young were eating solid food may well have been caused entirely by changes in the quantity rather than in the quality of milk produced by the mothers, and further, that one factor bringing about the relative decrease in the quantity of milk produced by the deficient mother may have been the failure of the deficient young to nurse out all their mother's milk because of their limited appetite. However, since in the previously described experiment involving injected young no significant difference was observed in the weight gain between uninjected and injected young during the nursing period, it would seem that B₁₂ deficiency must have also had a direct effect on the mothers in decreasing the total quantity of milk produced; had the effect of the B₁₂ deficiency been only upon the appetite of the B₁₂-deficient young, the injected young would presumably have obtained more milk than the uninjected young and hence gained more weight.

Food consumption during lactation

Table 7 shows the food consumption of B₁₂-deficient and B₁₂-supplemented mothers and their litters during lactation on B₁₂-deficient rations 192 and 443; these two rations were

TABLE 7

Food consumption of mothers and litters during the lactation period on B₁₂-deficient and B₁₂-supplemented rations

RATION FED DURING LACTATION		NO. OF LITTERS ¹	FOOD CONSUMPTION DURING							
No.	Supplement		1-7th day		8-14th day		15-21st day		22-25th day	
			gm	t	gm	t	gm	t	gm	t
Mothers transferred from stock to experimental ration at parturition										
192										
or										
443	None	10	20.8		31.2		39.3		49.3	
192	B ₁₂			0.1		0.6		0.7		1.4
or	(2 µg/day)									
443		11	20.9		32.0		40.9		53.0	
Mothers on experimental ration through previous lactation period(s):										
192										
or										
443	None	7	15.9		26.3		34.5		37.4	
192	B ₁₂			4.2**		2.8*		2.2*		4.9**
or	(2 µg/day)									
443		13	19.5		29.8		39.0		48.9	

¹ All of the litters included here whose mothers were transferred to the experimental rations at parturition contained 6 young each throughout the lactation period. All those whose mothers had been on the experimental ration through previous lactation periods contained 6 young each from the end of the first week of lactation to weaning; during the first 7 days of lactation, an average of 7.90 young were nursed by each mother in the B₁₂-deficient group and an average of 7.92 young were nursed by each mother in the B₁₂-supplemented group.

very similar and the weights of the young at comparable periods during lactation were essentially the same. By comparison with table 2 (ration 192), it can be seen that differences in food consumption of unsupplemented and supple-

mented mothers largely followed differences in weights of the young during the lactation period. Thus it would appear that, as might be expected, a decreased output of milk by the mothers was accompanied by a decreased intake of food by the mothers.

SUMMARY

The feeding of vitamin B₁₂-deficient casein-containing rations to mother rats during the lactation period resulted in smaller pre-weaning weights and post-weaning weight gains of the young, and in a decreased storage of B₁₂ in the organs of the young previous to the time that they began to eat solid food. Significantly lower pre-weaning weights of the young occurred more quickly when the B₁₂-deficient rations contained yeast than when they were yeast-free. A similar effect was noted when lactose-containing rations were compared to lactose-free rations. Retaining mothers over several generations on yeast-containing B₁₂-deficient rations, supplemented and unsupplemented with B₁₂, did not result in differences between pre-weaning weights of young or between post-weaning weight gains of young that were markedly greater than those that occurred in the parent generation.

Access to the B₁₂-fed mother's excreta was not found to be a significant factor in explaining differences in weights or weight gains between the young from B₁₂-deficient and those from B₁₂-supplemented mothers. It appeared that a decrease in the quantity of milk produced by B₁₂-deficient mothers was the primary factor in explaining the lower weight gains prior to the time the young began to consume solid food. The decreased milk output was accompanied by decreased food consumption by the deficient mothers.

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THE EFFECT OF DIET ON THE COMPARATIVE ACTIVITIES OF PYRIDOXAL, PYRIDOXAMINE AND PYRIDOXINE FOR CHICKS^{1, 2, 3}

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Most of the vitamin B₆ in natural foodstuffs is present as pyridoxal or pyridoxamine rather than as pyridoxine (Rabinowitz and Snell, '48). Despite this fact, most nutritional studies with this vitamin have employed pyridoxine. For this reason, additional data on the comparative activities for higher animals of these naturally-occurring forms of the vitamin are needed.

Pyridoxal, pyridoxamine and pyridoxine are equally active for rats and chicks when administered by injection or by mouth apart from other components of the ration (Snell and Rannefeld, '45; Sarma et al., '46a). The injected compounds are also equally active for the dog (Sarma et al., '46b). When mixed in synthetic rations high in soluble carbohydrates, pyridoxal and pyridoxamine are distinctly less active than pyridoxine for chicks (Sarma et al., '46a), rats (Sarma et al., '46a; Linkswiler et al., '51) and mice (Miller and Baumann, '45). With rats, the addition of aureomycin to such rations reduces the requirement for each of the forms of vitamin

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B₆; however, the effect is greater with pyridoxal and pyridoxamine, so that under these conditions the three forms of the vitamin are equally active (Linkswiler et al., '51). Addition of substantial amounts of methionine to such rations increases the requirement of rats for vitamin B₆, and pyridoxal and pyridoxamine equal or surpass pyridoxine in growth-promoting activity under these conditions (DeBey et al., '52).

It is clear that the relative activities of the forms of vitamin B₆ vary with the ingredients of the ration. In the present study, the effects of variations in the carbohydrate of the diet and supplementation with aureomycin on their activity for chicks were determined.

EXPERIMENTAL

The chicks used in this study were the progeny of New Hampshire males and Single Comb White Leghorn females fed diet B-1 (Robblee et al., '48). Ten or more chicks were used per group. They were kept in electrically heated batteries with raised screen floors for the experimental period of 4 weeks.

The basal ration, modified slightly from that of Luckey et al. ('45), had the following composition: carbohydrate 61, hot alcohol extracted casein 18, gelatin 10, salts V (Briggs et al., '43) 6, soybean oil 4, feeding oil (2,000A-300D) 0.5, choline chloride 0.2, inositol 0.1, and L-cystine 0.3 gm per 100 gm of ration. The following were added in milligrams per kilogram: α -tocopherol 3.0, thiamine hydrochloride 3.0, niacin 50, calcium pantothenate 20, biotin 0.2, menadione 0.5, folic acid 4.0, *p*-aminobenzoic acid 100, riboflavin 6.0 and vitamin B₁₂ 0.03. All of the rations were fed with and without 25 mg of aureomycin hydrochloride per kilogram of diet.

Pyridoxal, pyridoxamine or pyridoxine were added at equimolar levels as indicated in the tables. The rations were mixed at least every 10 days. Feed and water were supplied ad libitum.



RESULTS

With either glucose or autoclaved starch as the dietary carbohydrate, aureomycin was ineffective in stimulating growth at the low levels of pyridoxine (table 1). The marked sparing effect of aureomycin on the pyridoxine requirement

TABLE 1

Effect of aureomycin on the activity of pyridoxine with autoclaved starch or glucose as the dietary carbohydrate

LEVEL OF PYRIDOXINE · HCL PER GM OF DIET	AVERAGE WEIGHT OF CHICK AT 4 WEEKS ¹					
	Autoclaved starch				Glucose	
	Experiment 1		Experiment 2		Experiment 2	
	No aureo- mycin	Plus aureo- mycin	No aureo- mycin	Plus aureo- mycin	No aureo- mycin	Plus aureo- mycin
μg	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>	<i>gm</i>
0.25	66 ^s	74 ^r				
0.50	95 ^s	95 ¹	132 ^s	126 ^s	119 ⁴	110 ²
0.70	146	149 ³	146 ¹	152 ³	148	157
0.90	196	202	189	179 ¹	212 ¹	215 ¹
1.20	256	263	311	318	265	305 ¹

¹ Superscript indicates dead chicks. Twelve chicks started in experiment 1, 10 in experiment 2. Average day-old weight = 38 gm.

TABLE 2

Effect of feeding aureomycin with different carbohydrates and adequate vitamin B₆¹

CARBOHYDRATE	VITAMIN B ₆ ADDED		AVERAGE WEIGHT OF CHICK AT 4 WEEKS	
	Amount	Form	No antibiotic	Plus aureomycin
	$\mu g/gm$		<i>gm</i>	<i>gm</i>
Glucose	1.5	Pyridoxine · HCl	310	349
	2.0	Pyridoxine · HCl	330	341
Sucrose	4.0	Pyridoxine · HCl	266	309
	4.0	Pyridoxal · HCl	297	338
	4.7	Pyridoxamine · 2 HCl	246	288
Autoclaved starch	2.0	Pyridoxine · HCl	380	397
	2.0	Pyridoxal · HCl	378	396
	5.0	Pyridoxine · HCl	368	412
	5.0	Pyridoxal · HCl	356	387

¹ Experiments involving the different carbohydrates were not run concurrently. At least 10 chicks were used per group.

TABLE 3

Effect of carbohydrate source and aureomycin on the comparative activities of pyridoxal, pyridoxamine and pyridoxine

CARBOHYDRATE (%) AND VITAMIN B ₆ (μG/GM) IN DIET	GROWTH AND SURVIVAL ¹ AT 4 WEEKS			
	No aureomycin		Plus aureomycin	
	Av. wt.	Survival	Av. wt.	Survival
	gm		gm	
Glucose (61%)				
Pyridoxine · HCl (1.2)	231	40/46	270	44/46
Pyridoxal · HCl (1.2)	192	31/33	225	32/33
Pyridoxamine · 2 HCl (1.4)	180	25/33	212	28/33
Pyridoxine · HCl (0.70)	144	20/20	176	20/20
Pyridoxal · HCl (0.70)	137	16/20	150	15/20
Pyridoxamine · 2 HCl (0.82)	123	12/20	118	12/20
Glucose (41%) + lactose (20%)				
Pyridoxine · HCl (1.2)	288	19/20	332	19/20
Pyridoxal · HCl (1.2)	223	20/20	250	20/20
Pyridoxamine · 2 HCl (1.4)	177	16/20	212	16/20
Pyridoxine · HCl (0.70)	210	17/20	197	18/20
Pyridoxal · HCl (0.70)	170	11/20	174	11/20
Pyridoxamine · 2 HCl (0.82)	170	7/20	141	8/20
Sucrose (61%)				
Pyridoxine · HCl (1.2)	208	10/10	257	10/10
Pyridoxal · HCl (1.2)	129	8/10	141	9/10
Pyridoxamine · 2 HCl (1.4)	142	10/10	190	10/10
Sucrose (41%) + lactose (20%)				
Pyridoxine · HCl (1.2)	254	10/10	309	10/10
Pyridoxal · HCl (1.2)	223	9/10	199	9/10
Pyridoxamine · 2 HCl (1.4)	180	9/10	253	10/10
Autoclaved starch (61%)				
Pyridoxine · HCl (1.2)	305	30/30	316	29/30
Pyridoxal · HCl (1.2)	294	29/30	343	30/30
Pyridoxamine · 2 HCl (1.4)	284	29/30	335	30/30
Pyridoxine · HCl (0.70)	156	30/32	165	26/32
Pyridoxal · HCl (0.70)	143	26/32	154	26/32
Pyridoxamine · 2 HCl (0.82)	176	30/32	173	31/32
Autoclaved starch (41%) + lactose (20%)				
Pyridoxine · HCl (1.2)	301	10/10	344	10/10
Pyridoxal · HCl (1.2)	315	10/10	321	10/10
Pyridoxamine · 2 HCl (1.4)	307	10/10	343	10/10

¹Survival is expressed as the number of chicks surviving the 4-week test period divided by the number of chicks started. No group contained less than 10 birds. Multiples of that number represent the average of several groups run at different times.

noted with the rat (Linkswiler et al., '51) is thus not present with rations of this type in the chick. At the adequate levels of vitamin B₆ shown in table 2, the diet becomes complete with respect to known vitamins, and the familiar growth-promoting properties of the antibiotic then become apparent.

The effects of aureomycin and of variation in the carbohydrate on the comparative activities of pyridoxal, pyridoxamine and pyridoxine are shown in table 3. In confirmation of previous results (Sarma et al., '46a) pyridoxal and pyridoxamine are less active than pyridoxine in rations high in soluble carbohydrate, whether this is glucose or sucrose. Some stimulation of growth by the antibiotic is evident, but it exerts little or no effect on the comparative activities of the three forms of vitamin B₆, in contrast to the result found in rats (Linkswiler et al., '51). With autoclaved starch, the result is notably different. No statistically significant differences in the activities of the three compounds are observed, either in the presence or absence of the antibiotic. The results using autoclaved starch as the dietary carbohydrate do not confirm the report of Luckey et al. ('45) that pyridoxal and pyridoxamine are less active than pyridoxine. This difference may be due to the fact that their ration contained no added source of vitamin B₁₂.

The effects of additions of 20% of lactose to diets sub-optimal in vitamin B₆ are of considerable interest. Marked stimulation of growth resulted in those diets based on glucose or sucrose, but little or none resulted with autoclaved starch. The comparative activities of the three forms of vitamin B₆ were unchanged by this addition. Microbiological analysis of the lactose revealed that no vitamin B₆ was present.

DISCUSSION

Several conditions were cited in the introduction under which pyridoxal, pyridoxamine and pyridoxine showed equal activities in promoting growth of rats and chicks. The equal activities of the three compounds for chicks when diets based upon autoclaved starch are employed is another instance of

this behavior. It should be pointed out that this type of diet simulates natural diets much more closely than do those containing up to 61% of soluble carbohydrate. It is only on this latter type of diet that pyridoxal and pyridoxamine have proved less active than pyridoxine. Perhaps, therefore, this difference in activity does not exist under conditions of practical nutrition.

In explanation of the differences in activity observed on diets high in soluble carbohydrates, it was suggested (Sarma et al., '46a) that pyridoxal and pyridoxamine were utilized more readily than pyridoxine by the intestinal flora. When injected, or when fed apart from other food so that absorption was rapid, the three compounds should and do show equal activities. When mixed with the ration, absorption was considered as being delayed, so that bacteria, which either selectively destroy or utilize pyridoxal and pyridoxamine in preference to pyridoxine, compete with the host for these substances. One group of organisms for which this is demonstrably true are the lactic acid bacteria, which are among the most numerous in the gastrointestinal tract. They occur in substantial numbers in the duodenum and small intestine as well as in the colon (Shapiro and Sarles, '49; Shapiro et al., '49) and are greatly increased in the upper portion of the intestinal tract by feeding diets high in soluble carbohydrates (Johansson et al., '48). When diets based on starch are fed, the numbers of these organisms in the duodenum, but not in the lower portions of the tract, are decreased. The effects of various carbohydrates on the bacterial population of the upper portion of the intestinal tract, where the most active absorption of nutrients occurs, thus correlate with their effects upon the comparative activities of the forms of vitamin B₆, and lend support to the view that these differences result from a competition between bacterium and host for these essential nutrients.

The action of aureomycin in eliminating the observed differences in activity for the rat was interpreted (Linkswiler et al., '51) as indicating a relative decrease in population

of those organisms that compete with the host for vitamin B₆. According to the views developed above, this decrease need appear only in the numbers of microorganisms occurring in the upper portions of the intestinal tract, and not in the total count. Evidence for this selective decrease in numbers in the aureomycin-fed rat has recently appeared (Johansson et al., '52). Similar data for the chick are not available; however, the comparative activities of the three compounds in the chick are unchanged by the level of aureomycin used here. These differences in the rat and chick may reflect the recognized difference in composition of the intestinal flora of the two species (Evenson, '47), or other unidentified variables.

SUMMARY

1. Pyridoxal and pyridoxamine are less active than pyridoxine in supporting chick growth on otherwise complete rations containing large amounts (61%) of glucose or sucrose. The three compounds are equally active when autoclaved starch is the dietary carbohydrate. The latter type of ration simulates most closely those used in practical nutrition. The comparative activities of the three compounds on these rations is unchanged by feeding aureomycin at a level of 25 mg per kilogram of diet.

2. Substantial increases in growth with diets suboptimal in vitamin B₆ resulted when 20% of lactose was added to diets based on glucose or sucrose. No such increase resulted from addition of lactose to the starch-containing diet. The comparative activities of the three forms of vitamin B₆ are not changed by addition of this amount of lactose to any of these diets.

3. Possible explanations for the observed differences in activity of pyridoxal, pyridoxamine and pyridoxine with the composition of the ration are considered. The results are consistent with known changes in composition of the intestinal flora, and indicate that competition of intestinal flora with the host for limited supplies of these nutrients may explain

the differences in relative activities of these compounds that occur with differences in the diet or mode of administration.

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EFFECT OF TERRAMYCIN AND CERTAIN
PHENYLARSONIC ACID DERIVATIVES ON THE
GROWTH AND INTESTINAL FLORA
OF TURKEY POULTS

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Morehouse and Mayfield ('46) reported that 3-nitro-4-hydroxyphenyl arsonic acid had growth-stimulating properties for chickens and for turkeys. Bird et al. ('48) confirmed this observation in chickens and found that phenylarsonic acid and *p*-hydroxyphenyl arsonic acid were comparable in their activity to 3-nitro-4-hydroxyphenyl arsonic acid. *M*-nitrophenyl arsonic acid also showed some growth-promoting activity while *p*-chlorophenyl arsonic acid, sodium arsenate, *m*-nitrobenzene sulfonic acid, salicylic acid, 3-nitrosalicylic acid and sodium *p*-phenolsulfonate were inactive. Morehouse ('49) found the optimum concentration of 3-nitro-4-hydroxyphenyl arsonic acid in the feed of chickens to be about 0.009%. The latter worker also noted that feed was utilized more efficiently by turkeys receiving 3-nitro-4-hydroxyphenyl arsonic acid than by their controls.

It has been amply demonstrated that certain antibiotics stimulate the growth of chicks and poults. Davis and Briggs ('51) have reviewed the early literature in this connection. These authors found that the chick and poult showed improved feed efficiency when the diet was supplemented with an antibiotic and stated that there was no indication that growth stimulation occurred as a simple manifestation of increased feed consumption. On the other hand, it had been

suggested earlier by the data of Scott and Glista ('50) that under their conditions aureomycin and 3-nitro-4-hydroxy-phenyl arsonic acid stimulated growth in chicks through the medium of increased feed consumption.

Many workers have shown that diet influences the intestinal microflora of chickens. Johansson et al. ('48) observed a change in the fecal microflora associated with dietary carbohydrate in pullets. The bacterial types reported were coliforms, enterococci and lactobacilli. Their data also suggested that anaerobic lactobacilli exist in the intestines of chickens in considerable numbers. Moore et al. ('46) found that orally administered streptomycin brought about a reduction in coliform bacteria in the cecal contents of chicks. Elam et al. ('51) reported that feeding penicillin caused a significant increase in total numbers of intestinal microorganisms as indicated by thioglycollate counts. This antibiotic also caused a significant increase in enterococci. The administration of penicillin either orally or intravenously resulted in a marked increase in penicillin resistant organisms in the intestinal tract. Anderson et al. ('52) reported that dietary penicillin effected a reduction in the pH of the cecal contents, which was associated with an increase in numbers of lactobacilli, aciduric, anaerobic and microaerophilic, aerobic and coliform types of organisms. The increase in coliforms was due to the presence of an atypical strain of *E. coli*. This strain lacked the characteristic green metallic sheen, fermented lactose slowly and did not attack dulcitol. Enterococci counts were reduced by the addition of penicillin to the diet.

The present experiment was made to determine the influence of terramycin and certain phenylarsonic acid derivatives on the growth and cecal flora of turkey poults.

MATERIALS AND METHODS

Day-old Broad Breasted Bronze female poults were weighed individually and distributed into experimental groups on the basis of weight. Each group was comprised of 18 poults.

The birds were reared in electrically heated battery brooders with raised wire floors.

The percentage composition of the basal diet was as follows: corn 16, wheat 19, oat groats 5, dehydrated alfalfa 2, soybean oilmeal (44% protein) 50, limestone 1.5, steamed bone meal 5.0, iodized salt 0.5, fish oil (2,400 A and 400 D per gram) 1.0. In addition, to each 100 lb. of diet the following were added: manganese sulfate (technical) 7 gm, riboflavin 0.2 gm, niacin 0.5 gm, and vitamin B₁₂ 0.1 mg. When included, terramycin hydrochloride was present to the extent of 15 mg per kilogram, and the various phenylarsonic acid derivatives in the amount of 70 mg per kilogram of diet. The experimental diets and water were supplied ad libitum.

The birds were weighed individually at 21 and 30 days of age and the feed consumption was recorded. Two birds of average weight were removed from each group after every weighing and sacrificed for bacteriological analysis of the cecal contents. They were aseptically opened and the ceca ligatured off. The outer surface was then washed with a mercuric chloride solution and removed to sterile petri dishes. The cecal contents were expressed by the use of a sterile spatula and forceps into a second series of sterile petri dishes.

The pH of the various samples was recorded using a Beckman pH meter and employing contact electrodes applied to the thoroughly mixed samples. A 1-gm sample of the cecal contents was added to a 99-ml sterile distilled H₂O blank containing glass beads. The sample was thoroughly shaken on an automatic shaking machine for 5 minutes. From this initial dilution, dilutions of 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, and 10⁻⁹ were made and duplicate 1-ml samples of each transferred to the media indicated below.

Levine's eosin methylene blue agar was employed for the enumeration and differentiation of the members of the coliform group.

"S. F." broth of Hajna and Perry ('43) dispersed in 10 ml amounts in test tubes was used for the cultivation of en-

terococci. The M. P. N. method was used for the enumeration of this group of microorganisms.

Tomato juice agar (Difco) was chosen for plating the samples for the isolation and counting of lactobacilli. The plates were overlaid with 2% agar to reduce O_2 tension.

Tryptone glucose extract agar with 2% yeast extract (Difco) was used for the cultivation and enumeration of aerobes. This same medium with 1 ml per liter of a 1.6% alcoholic solution of Brom Cresol Purple and 2% skim milk was used for the cultivation and enumeration of the aciduric and proteolytic types of organisms.

Linden's thyoglycollate medium (Difco) was placed in test tubes in 10 ml amounts for the cultivation of anaerobes. The M. P. N. method was used for counting these organisms. A 2% agar solution was added after inoculating the tubes; this formed a plug and helped prevent reabsorption of O_2 . The above media were incubated at 37°C. for 48 and 72 hours with the exception of the "S. F." medium which was incubated at 41°C. for 72 hours. Viable bacterial counts were recorded following incubation. From the plates representing the highest dilutions which supported the growth of a minimum of 30 colonies, representative colonies were "fished" off and maintained on either tryptone glucose yeast extract agar or tomato juice agar slants as stock cultures. A total of 225 cultures was selected on the basis of their colony characteristics. These cultures were examined as soon as possible after isolation, with a minimum of transferring in order to prevent form variation. Cultures were studied for colony characteristics and Gram reaction. Any mixed cultures observed were restreaked and isolated colonies picked off in order to obtain pure cultures.

RESULTS

Observations of growth

The weight and feed efficiency data are presented in table 1. The final weight data were examined statistically by the

TABLE 1
Effect of terramycin and certain phenylarsonic acid derivatives on the growth of turkey poults

GROUP NO.	PHENYLARSONIC ACID DERIVATIVE	TERRA-MYCIN HCl	AGE — 21 DAYS		AGE — 30 DAYS		TOTAL FEED CONSUMED
			Weight	Feed/Gain	Weight	Feed/Gain	
			gm		gm		lb.
1	None	—	271 (18) ¹	2.00	494 (16) ¹	2.08	34.25
2	None	+	341 (18)	1.91	585 (16) ²	2.05	41.00
3	Magnesium 4-hydroxyphenylarsonate	—	310 (18)	1.90	550 (16) ²	2.04	38.00
4	Magnesium 4-hydroxyphenylarsonate	+	325 (18)	1.84	568 (16) ²	2.02	39.00
5	3-amino 4-hydroxyphenyl arsonic acid	—	287 (18)	2.00	522 (16)	2.12	37.25
6	3-amino 4-hydroxyphenyl arsonic acid	+	330 (17)	1.93	596 (15) ²	2.13	40.50
7	3-acetyl amino 4-hydroxyphenyl arsonic acid	—	276 (18)	1.99	506 (16)	2.20	37.25
8	3-acetyl amino 4-hydroxyphenyl arsonic acid	+	343 (18)	1.88	617 (16) ²	1.88	39.75
9	3-nitro 4-hydroxyphenyl arsonic acid	—	312 (18)	1.85	560 (16) ²	1.99	37.75
10	3-nitro 4-hydroxyphenyl arsonic acid	+	335 (18)	1.82	590 (16) ²	1.98	39.75

¹ Figures in parentheses indicate number of birds surviving.

² Weight significantly greater than group 1 ($P < 0.01$).

method of analysis of variance. An "F" value was obtained indicating highly significant differences due to diets. Comparing the mean square with the error mean square gave a measure of the significance of the differences between means. It will be noted that additions to the basal diet of terramycin alone or in combination with any of the phenylarsonic acid derivatives resulted in weight increases which were highly significant at 30 days of age. In the absence of terramycin highly significant weight increases resulted from the inclusion of magnesium 4-hydroxyphenylarsonate and 3-nitro-4-hydroxyphenyl arsonic acid. The weight increases caused by the 3-amino- and 3-acetylamino-derivatives were not significant at $P = 0.05$. In general, significant increases in weight were accompanied by slight improvement in feed efficiency.

The weight increases caused by adding terramycin to diets containing magnesium 4-hydroxyphenylarsonate or the 3-nitro-derivative were not significant. On the other hand, considered on the basis of both weight and feed efficiency there appears to be a slight advantage in favor of combining either of these compounds with the antibiotic at the levels employed. In addition, while the mean weight of the group fed the 3-acetylamino-derivative in the presence of terramycin was not significantly greater than that of the group fed antibiotic alone, when feed efficiency is also considered, there appears to be a synergistic relationship between terramycin and the acetylamino-derivative.

On the basis of the growth-promoting activity in chicks of phenylarsonic acid and its nitro- and hydroxy-derivatives, Bird et al. ('48) concluded that the presence of the nitro and hydroxy groups was not essential. In the present experiment the 3-nitro-derivative both in the absence and the presence of terramycin, resulted in slightly (but not significantly) greater weight and somewhat better feed efficiency than the magnesium 4-hydroxyphenylarsonate. This suggests that, at the levels used, the latter compound was somewhat inferior to the former. The lack of significant response to the 3-amino- and 3-acetylamino-derivatives suggests

that the presence of these substances reduced the effectiveness of the compound in the absence of antibiotic.

Bacteriological findings

The results of the pH measurements and viable cecal floral counts are presented in tables 2 and 3. Each value represents the average of determinations made on two birds. It will be noted that, at both 21 and 30 days of age, terramycin either alone or in combination with the various phenylarsonic acid derivatives effected a lowering of the pH of the cecal contents. In the absence of antibiotic the phenylarsonic acid derivatives, with one exception, caused a decrease in the pH of the cecal contents. The addition of terramycin in the presence of the various phenylarsonic acid derivatives, again with a single exception, resulted in a lower pH than with the phenylarsonic acid derivative alone.

In most cases, aerobic counts were reduced by additions to the basal diet of terramycin, phenylarsonic acid derivatives or combinations thereof. However, the 3-amino-derivative, and the 3-nitro-derivative in the presence of terramycin, caused an increase in aerobes. This group of organisms was made up of the following types: (a) Gram-negative short rods occurring singly, facultative and motile; (b) Gram-negative coccoid forms to long rods—single or in pairs, non-motile, non-spore-forming and facultative; (c) Gram-negative long rods—single, non-motile, non-spore-forming and facultative. Variation in numbers of aerobes due to diet was caused mainly by differences in organisms belonging to (b).

Anaerobic counts were higher than aerobic counts. Terramycin, phenylarsonic acid derivatives and combinations of these materials caused a reduction in anaerobic counts at 21 days of age. At 30 days of age, the influence of the dietary supplements on the counts of these organisms was variable. The following types of organisms were observed in this group: (a) Gram-positive cocci in chains and masses—non-motile, non-spore-forming and microaerophilic; (b) Gram-negative short, fat rods with rounded ends—single and in pairs, non-

TABLE 2
Effect of terramycin and certain phenylarsonic acid derivatives on the cecal microflora of 21-day-old poult
 (Counts indicate numbers of organisms per gram of cecal contents)

GROUP NO.	PHENYLARSONIC ACID DERIVATIVE	pH CECALE CONTENTS	TERRA-MYCIN HCl	AEROBES ($\times 10^5$)	ANAEROBES ($\times 10^5$)	ACIDURIC ($\times 10^6$)	PROTEOLYTIC ($\times 10^5$)	LACTOBACILLI ($\times 10^5$)	COLIFORMS ($\times 10^5$)	ENTEROCOCCI ($\times 10^4$)
1	None	6.20	—	30.0	12.0	2.1	91.0	5.0	24.0	11.0
2	None	5.05	+	1.7	1.8	1.2	5.0	22.0	13.2	10.0
3	Magnesium 4-hydroxy-phenylarsonate	5.85	—	1.0	0.2	1.0	0.3	2.0	31.4	13.0
4	Magnesium 4-hydroxy-phenylarsonate	5.75	+	1.6	< 0.0	1.1	5.0	220.0	10.3	11.0
5	3-amino 4-hydroxy-phenyl arsonic acid	5.80	—	38.0	1.7	34.0	40.0	30.0	28.0	14.0
6	3-amino 4-hydroxy-phenyl arsonic acid	5.50	+	1.2	0.5	0.6	6.0	5.0	5.2	100.0
7	3-acetyl amino 4-hydroxy-phenyl arsonic acid	6.35	—	2.0	0.2	1.0	10.0	16.0	13.2	150.0
8	3-acetyl amino 4-hydroxy-phenyl arsonic acid	5.80	+	15.0	< 0.0	12.0	30.0	16.0	12.0	149.0
9	3-nitro 4-hydroxy-phenyl arsonic acid	5.75	—	1.1	1.4	1.5	0.5	10.0	11.0	10.0
10	3-nitro 4-hydroxy-phenyl arsonic acid	5.95	+	111.0	1.1	107.0	40.0	220.0	35.0	132.0

TABLE 3
Effect of terramycin and certain phenylarsonic acid derivatives on the cecal microflora of 30-day-old poult
 (Counts indicate numbers of organisms per gram of cecal contents)

GROUP NO.	PHENYLARSONIC ACID DERIVATIVE	pH CECAL CONTENTS	TERRAMYCIN HCl	AEROBES ($\times 10^6$)	ANAEROBES ($\times 10^7$)	ACIDURIC ($\times 10^6$)	PROTEOLYTIC ($\times 10^5$)	LACTOBACILLI ($\times 10^6$)	COLIFORMS ($\times 10^6$)	ENTEROCOCCI ($\times 10^4$)
1	None	5.95	—	7.3	0.1	7.2	1.0	9.0	0.5	1.0
2	None	5.70	+	3.5	0.9	3.3	2.0	34.0	0.1	11.0
3	Magnesium 4-hydroxyphenylarsonate	5.65	—	2.6	< 0.0	2.6	0.4	12.0	0.7	13.1
4	Magnesium 4-hydroxyphenylarsonate	5.40	+	1.9	10.0	12.0	7.2	101.0	30.0	500.0
5	3-amino 4-hydroxyphenylarsonic acid	5.65	—	12.0	2.0	10.0	20.0	5.0	247.0	25.0
6	3-amino 4-hydroxyphenylarsonic acid	5.50	+	5.5	0.7	5.3	2.0	14.0	2.6	19.0
7	3-acetylaminio 4-hydroxyphenylarsonic acid	5.85	—	3.9	< 0.0	3.9	0.2	0.7	0.1	31.0
8	3-acetylaminio 4-hydroxyphenylarsonic acid	5.60	+	0.4	< 0.0	0.4	0.3	160.0	8.9	29.1
9	3-nitro 4-hydroxyphenylarsonic acid	5.50	—	4.3	10.0	4.1	2.0	39.0	54.0	0.1
10	3-nitro 4-hydroxyphenylarsonic acid	5.50	+	11.5	0.1	11.1	4.0	16.0	31.0	1.9

spore-forming, non-motile and microaerophilic. The numbers of the different types did not appear to be consistently related to the diet.

The aciduric types of organisms were increased with certain supplements and decreased with others. This group comprised Gram-negative cocci, short rods and long rods. These were non-spore-forming and aerobic. Proteolytic organisms were much less numerous at 30 days of age than at 21 days of age. At 21 days all dietary treatments caused a reduction in proteolytic counts while at 30 days of age this trend was by no means consistent. The proteolytic types of organisms were Gram-negative cocci and short rods, occurring singly. All these organisms were non-motile and facultative with the rods being spore-formers.

The lactobacilli counts at 21 days of age were increased, in most cases, by the addition of terramycin, phenylarsonic acid derivatives or combinations of these compounds. However, in the case of the diet containing magnesium 4-hydroxyphenylarsonate in the absence of antibiotic, and that containing the 3-amino-derivative in the presence of antibiotic, these counts were not increased. It will be noted that, at 30 days of age, only in the case of the 3-amino-derivative and the 3-acetylamino-derivative, in the absence of terramycin, were the lactobacilli counts not increased as compared to the basal diet. The lactobacilli isolated from tomato juice agar appeared as pin-point colonies and grew below the surface. The cells were Gram-positive rods occurring singly, in pairs or as long chains. They exhibited varying degrees of pleomorphism and displayed granulation when Gram-stained. The age and amount of culturing influenced the morphology of the cells. The cells were non-motile and microaerophilic. Pleomorphism was more marked in the cells from birds receiving terramycin or the phenylarsonic acid derivatives, or both, than in cells isolated from birds fed the basal diet. Considerable difficulty was experienced in maintaining the strains which showed variation.

The data concerning coliform counts indicate that these organisms were influenced by dietary treatments. The effect was greater at 30 days than at 21 days of age. In some instances the treatment tended to decrease the coliform counts and in other cases there appeared to be marked increases in the numbers of these organisms as a result of the supplements used. The coliforms were Gram-negative short rods or coccoid to long rods. They occurred singly, were non-spore-forming, motile or non-motile and facultative. In the presence of either terramycin or phenylarsonic acid derivatives, or both, the coliforms differed from those found in birds fed the basal diet in that many of the former failed to give a normal Imvic's reaction, fermented lactose slowly, did not attack dulcitol and failed to give the same characteristic green metallic sheen.

At 21 days of age certain dietary treatments caused an increase in enterococci organisms while others appeared to bring about little or no change. At 30 days of age the enterococci were increased by all diets excepting those containing the 3-nitro-derivative. This group of organisms consisted of Gram-positive coccoid forms. The cells were spherical in shape and occurred in pairs of short chains. They were non-motile, facultative anaerobes.

DISCUSSION

A point of interest in this work is the relatively similar influence of terramycin and certain phenylarsonic acid derivatives on the cecal floral counts and on the pH of the cecal contents. It is also noteworthy that increases in weight were accompanied in most cases by a reduction in pH of the cecal contents. The reduction in pH caused by the 3-acetyl-amino-derivative in the absence of antibiotic was less than that resulting from supplementation with the other phenylarsonic acid derivatives and the former compound failed to produce a significant growth response. In addition, the decreases in pH resulting from the inclusion of terramycin in diets containing the various phenylarsonic acid derivatives

were accompanied by weight increases — albeit not significant in all cases. These results suggest that the reduction in pH of the cecal contents may be associated with the growth stimulation brought about by terramycin and phenylarsonic acid derivatives.

While most dietary treatments caused a reduction in aerobes accompanied by an increase in growth, birds fed the diet containing the 3-nitro-derivative and terramycin showed an increase in these organisms, and a significant increase in growth. Furthermore, the 3-acetylamino-derivative in the absence of antibiotic caused a marked reduction in aerobes and no significant increase in growth. These results suggest that changes in aerobic counts are not necessarily correlated with growth.

The decrease in numbers of anaerobes at 21 days of age, caused by dietary treatment, did not appear to be closely associated with changes in growth. Similarly, the counts of these organisms varied widely at 30 days of age and appeared to bear little relationship to growth changes. It would also appear that changes in numbers of aciduric, proteolytic, coliform and enterococci organisms were not consistently involved in the growth-promoting effect of terramycin and phenylarsonic acid derivatives.

With respect to lactobacilli, at 30 days of age only in the case of the diets containing the 3-amino-derivative and the 3-acetylamino-derivative, in the absence of antibiotic, were the counts of this group of organisms not increased as compared to the basal diet. Furthermore, these were the only dietary treatments which did not result in a significant improvement in weight. Another interesting point is that the diet containing the 3-acetylamino-derivative in combination with terramycin resulted in the highest lactobacilli count and the greatest numerical weight. While there were two exceptions to an increase in weight being accompanied by a greater number of lactobacilli organisms at 21 days of age, the data as a whole suggest that the weight increases caused by ter-

ramycin and phenylarsonic acid derivatives were associated with increased counts of lactobacilli.

SUMMARY

Highly significant increases in the weight of female turkey poults were obtained by inclusion in the diet of magnesium 4-hydroxyphenylarsonate and 3-nitro-4-hydroxyphenyl arsonic acid.

The weight increases caused by including 3-amino-4-hydroxyphenyl arsonic acid and 3-acetylamino-4-hydroxyphenyl arsonic acid were not significant.

Additions to the basal diet of terramycin alone or in combination with any of the phenylarsonic acid derivatives resulted in highly significant increases in weight. For the most part, weight increases were accompanied by a slight improvement in feed efficiency.

The increases in weight caused by terramycin, phenylarsonic acid derivatives or combinations thereof, were accompanied in most cases by a reduction in the pH of the cecal contents.

Terramycin, phenylarsonic acid derivatives and combinations of these materials markedly influenced the cecal flora. The effects of the antibiotic and some of the phenylarsonic acid derivatives were similar in many respects.

In general, weight increases were accompanied by increases in the lactobacilli counts of the cecal contents. The numbers of aciduric, proteolytic, coliform and enterococci organisms were not consistently in agreement with increases in weight.

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THE RELATION OF THE RATE OF GROWTH TO THE DIET. A STUDY OF A STOCK COLONY RATION FOR ALBINO RATS

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INTRODUCTION

In the management of an animal colony, the selection of the breeding stock and of the diet are important. As is well known, a colony of albino rats has been maintained at The Connecticut Agricultural Experiment Station in New Haven since 1910. In that year Osborne and Mendel obtained animals from a local pet shop and two years later a few extracted albinos were bought from Dr. Donaldson at the Wistar Institute. This original colony supplied rats for a similar one which was maintained for many years in the Department of Physiological Chemistry at Yale and during that period the strain became known as the "Yale Strain."

Inevitably, during the 40 years the colony has been in existence, there have been a few changes in the type of ration used. Mendel and Hubbell ('35) recorded the rate of growth and breeding performance of the animals at 4 intervals during the first 25 years and showed that an enhanced growth rate, which was first noted in 1931, could be attributed to the type of food supplied and not to the selection of unusually large animals for breeding. Similar data for each of the 15 years since that report was published show that there has been a marked uniformity in the weights of the young at weaning and that the average weaning weights for both males and females for the entire period are identical with

the 1935 values. However, there has been a decline in the percentage of litters born and weaned. For the year 1935, 93% of the matings were fertile and 90% of the litters were weaned. In 1951 the corresponding values were 79 and 84%, respectively. In addition to these changes, performance has been rather unsatisfactory in some other respects. A few mothers kill their young, many males are infertile, and respiratory disorders persist. In general it has been observed that these departures from normal are transitory and may possibly be attributable to variations in the composition of the natural foods employed.

Because these fluctuations as well as the general downward trend observed in recent years might indicate the need for a modification of the stock ration, a small scale breeding experiment has been carried through the F_4 generation. Rats fed the colony ration of "Calf Meal Food" (Mendel and Hubbell, '35) have been compared with those in two other groups in which this diet was supplemented in one case with 3% whole liver substance¹ and in the other with 5 ml of orange juice² per 50 gm of rat per day. Liver substance was selected because it supplies vitamin B_{12} and perhaps others factors not yet identified, and in addition furnishes valuable animal protein. In connection with studies carried out on the vitamin content of orange juice (Krehl and Cowgill, '50), and because of the possible role of ascorbic acid in the nutrition of the rat under certain circumstances (Mayer and Krehl, '48), it seemed of interest to test the effect of the addition of this product as a supplement to the diet.

EXPERIMENTAL

To initiate the experiment, 18 female and 6 male weanling rats were assigned to each of three groups started, for laboratory convenience, at monthly intervals. Each group was subdivided into three groups of 6 female and two male rats

¹ Whole liver substance, Wilson and Co., Chicago, Ill.

² Reconstituted from the frozen juice, purchased from a wholesale distributor as a uniform lot.

on the basis of the food to be tested. Between the ages of 110 and 120 days they were mated according to the regular procedure in this laboratory, namely three females and one male in each cage. At the end of three weeks the females were transferred to separate cages. All litters were reduced at birth to 8 animals and the young were weaned at 21 days of age. If no litter was born, the female was remated with a different male. There were no further matings of those females that cast young but failed to wean them.

RESULTS AND DISCUSSION

There was somewhat more rapid growth of male rats fed the liver supplement in comparison with the controls or those that received the orange juice. This was true in all generations and there were no consistent changes from one generation to another. Male rats in the liver groups attained an average weight of 421 ± 20^3 gm at 110 days of age in contrast to 376 ± 31 gm for the controls and 350 ± 44 gm for those in the orange juice groups. The average weights of the females were essentially the same in all three groups, namely 251 ± 13 gm, 247 ± 14 gm, and 239 ± 18 gm, respectively, at 110 days. It is of interest to note the high variability in the weights of the males of the orange juice groups.

The reproduction data for the entire experiment are summarized in table 1. With respect to the percentage of litters born and weaned and the number of young born and weaned in each litter, the addition of liver substance or of orange juice had no effect. However, the average weaning weights of the rats in the orange juice group were markedly lower than those of the other two groups. For weanling females the *highest* value in the orange group was 43.8 ± 2.6 gm in the F_4 generation while the *lowest* in either of the other two groups was 46.7 ± 3.2 gm in the F_0 generation of those fed corresponding values were 45.9 ± 3.3 in the F_2 generation the diet supplemented with liver substance. For males the

³ Standard deviation.

in contrast to 47.9 ± 3.8 in the F_0 generation of the control group. No record was kept of food intake so that it is impossible to determine whether these differences in weaning weights can be associated with the consumption of rather large amounts of orange juice and a resultant lowered food intake for the animals of this group. The orange juice was

TABLE 1

The effect on reproduction of the addition of liver substance or orange juice to a stock ration for rats

DIET	GENER- ATION	MAT- INGS ¹	LITTERS BORN	YOUNG BORN PER LITTER	LITTERS WEANED	YOUNG WEANED PER LITTER	AVERAGE WEANING WEIGHTS	
							♀	♂
			%		%		gm	gm
Control	F_0	21	76.2	11.8	68.8	6.6	47.8 ± 4.7	47.9 ± 3.8
	F_1	21	71.4	8.2	73.3	6.4	48.5 ± 4.2	50.0 ± 2.9
	F_2	19	78.9	8.4	86.7	6.2	50.0 ± 3.9	54.0 ± 5.1
	F_3	24	66.7	8.8	75.0	6.3	49.1 ± 4.0	50.7 ± 4.2
	F_4	19	84.2	8.9	68.8	7.0	48.9 ± 4.0	50.9 ± 4.6
Control + liver	F_0	21	76.2	10.5	81.3	7.5	46.7 ± 3.2	50.4 ± 1.4
	F_1	20	85.0	9.2	82.4	7.1	48.0 ± 4.5	50.5 ± 3.1
	F_2	21	81.0	6.8	58.8	6.5	48.9 ± 2.3	49.2 ± 1.8
	F_3	20	75.0	8.4	86.7	6.8	48.5 ± 3.8	51.4 ± 3.7
	F_4	25	52.0	7.8	61.5	7.3	48.3 ± 2.1	50.3 ± 2.0
Control + orange juice	F_0	24	66.7	10.1	87.5	6.6	43.2 ± 2.6	44.5 ± 4.2
	F_1	22	81.8	8.8	66.7	6.7	42.2 ± 4.7	43.6 ± 3.7
	F_2	20	80.0	9.6	62.5	7.1	42.9 ± 4.0	45.9 ± 3.3
	F_3	22	72.7	9.9	75.0	7.0	42.3 ± 6.3	45.6 ± 4.0
	F_4	23	73.9	8.4	76.5	6.3	43.8 ± 2.6	43.0 ± 2.8

¹ Matings in excess of 18 (6 for each group) indicate rematings that were necessary to obtain a litter.

supplied during the day and water and food were withheld until the juice had been consumed. In a very few cases the rats failed to drink the entire portion of juice.

It is interesting to compare the data from this brief experiment with the records of the entire colony for the past 15 years. The average percentage of litters born and weaned is definitely lower in all of the experimental groups than it is in the main colony. In the experimental groups about

75% of the females produced young and slightly less than 75% of the litters were weaned. This is in contrast to 83 and 87% for the larger group and is also below the record for the most recent single year, 1951. The poorer results in the experimental groups may be associated with the fact that animals for new generations were always selected from first litters, whereas in the main colony the progeny of second and third matings are used almost entirely and only occasional use is made of young from first matings. However, although fewer young were born and weaned than in the main colony, the weaning weights of the controls and of the liver-fed rats were slightly above the 15-year average of 47.1 gm for females and 48.5 gm for males.

SUMMARY

In an attempt to effect an improvement in the breeding ration for albino rats the standard stock ration for the colony, "Calf Meal Food," was supplemented with 3% whole liver substance or with 5 ml of orange juice for each 50 gm of rat per day. Breeding data were secured through the F_4 generation. In general it was found that in the group fed the orange juice supplement there was a decreased rate of growth during the lactation period, due possibly to a lowered food intake. The weaning weights of 21-day-old rats were markedly below those of the other two groups. The post-weaning growth of male rats fed the liver-supplemented food was somewhat more rapid than it was in either of the other two groups. There was no difference between the groups with respect to the percentage of fertile matings or of litters weaned, but in all groups these results were less satisfactory than in the main colony during the same period of time.

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Nominations are solicited for the 1953 Award and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the Jury of Award may recommend that it will be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

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*Department of Biological Chemistry
University of Utah Medical School
Salt Lake City, Utah*

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Nominations are invited for the Osborne and Mendel Award of \$1000.00 established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

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The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

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*Department of Biochemistry and Nutrition
Duke University School of Medicine
Durham, North Carolina*

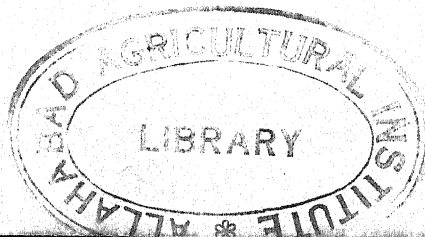
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